

# **Sonic hedgehog signalling in chronic renal allograft nephropathy and peripheral immunity**

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A thesis submitted for the degree of Doctor of Philosophy

University of Edinburgh, 2005  
Edinburgh University Medical School



## Abstract

The highly conserved Sonic hedgehog (Shh) signalling pathway has been shown to play an important role in the determination of cell fate and proliferation during development. Whilst the role of Shh signalling in the embryo has become increasingly well understood, the possible roles for Shh signalling in the adult are less clear. Recent reports in the literature have lent support to roles for Shh signalling in both remodelling processes and the maintenance of peripheral immunity.

Chronic renal allograft nephropathy (CAN) is a major cause of long-term renal failure in kidney allograft recipients. It is characterised by interstitial fibrosis, tubular atrophy, vasculopathy and a mononuclear cell infiltration. The inflammatory cell infiltrate consists of mainly macrophages and T cells, and macrophage infiltration and activity correlate directly with graft survival. Transforming Growth Factor Beta (TGF- $\beta$ ) and Vascular Endothelial Growth Factor (VEGF) are among a number of factors produced by activated macrophages and both growth factors have been demonstrated to be upregulated in CAN. That Shh signalling may play a role in CAN is suggested by recent suggestions in the literature that the Shh signalling pathway expression is upregulated in other fibrotic conditions, notably idiopathic pulmonary fibrosis and in murine models of pulmonary fibrosis. Furthermore, both TGF- $\beta$  and VEGF have been shown to be upregulated by Shh signalling. Thus, the first hypothesis central to this thesis was that Shh signalling pathway components are expressed in and relevant to normal and CAN kidney. The results presented demonstrate that, although Shh is expressed in normal renal epithelium, this expression is lost in CAN, a finding in contrast to observations in models of lung fibrosis. Possible reasons for this loss of expression are further investigated using cultures of a renal tubular epithelial cell line (ACHN) and primary cultures of murine epithelial cells.

Another recently identified area in which Shh signalling may be important in the adult is peripheral immunity, with recent reports demonstrating that the addition of recombinant Shh to cultures of activated CD4<sup>+</sup> T cells modulates the proliferation



and cytokine effector function of these cells. Furthermore, that macrophages express the receptor for Shh, Ptc, and that certain macrophage products including TGF- $\beta$  and VEGF have been shown to be upregulated by Shh signalling lends support to a possible role for Shh signalling in macrophages, an area which has, to date, not been explored. Thus, the second hypothesis underlying the work presented in this thesis was that Shh signalling plays a role in peripheral immunity. In particular, the role of Shh signalling in macrophages and CD4<sup>+</sup> T cells has been investigated. The findings presented suggest that recombinant Shh modulates the cytokine effector function of macrophages but that the predominant effects observed are most likely to be due to low levels of endotoxin contamination present within the recombinant proteins utilised to explore the effects of Shh *in vitro*. Shh also modulated the cytokine effector function, but not the proliferation of activated CD4<sup>+</sup> T cells, a finding partly in keeping with previous reports in the literature. However, in contrast to previous reports, blockade of Shh signalling failed to abrogate either the proliferation or cytokine effector response of activated CD4<sup>+</sup> T cells. Possible reasons for this disparity are discussed. Taken together, the findings from these investigations highlight possible roles for Shh signalling in adult kidney and peripheral immunity but emphasize the need for good quality, commercially available reagents to investigate the pathway. The current lack of such reagents precludes firm conclusions regarding possible roles for Shh signalling in peripheral immunity and the kidney or, indeed, roles relevant to the inflammatory processes seen in CAN.

## **Declaration**

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All work presented in this thesis was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Sonia Jane Wakelin

## Acknowledgements

Firstly, I am grateful to Professor James Garden and Mr John Forsythe from the Edinburgh Royal Infirmary Department of Surgery and Transplant Unit. Without their interest and support, this PhD would not have been possible. I am also grateful to Dr Sarah Howie whose patience, moral support and tireless optimism as a supervisor have been invaluable throughout this PhD. I would like to thank Professor Jonathan Lamb for his enthusiasm, encouragement and ideas during the early part of my PhD and I would also like to thank those who have provided financial support for the work presented in this thesis. Specifically in this regard, I would like to thank Edinburgh Royal Infirmary Department of Surgery and Transplant Unit whose funding initiated this work; the Royal College of Surgeons of Edinburgh; the Chief Scientist Office and the Wellcome Trust for supporting me as a Wellcome Trust Clinical Research Training Fellow.

Many thanks are due to all those in the MRC Centre for Inflammation Research who have been able to offer a wealth of experience and invaluable advice throughout this PhD. I am grateful to all those who have offered technical advice and, specifically, to Shonna McCall for her invaluable flow cytometry and cell sorting expertise.

Last, but by no means least, I would like to thank Kevin for his love, support and patience throughout this PhD. I would also like to thank my parents for their love and encouragement and Cleo and Lucy for their moral support.

*"We can judge our progress by the courage of our questions and the depth of our answers, our willingness to embrace what is true rather than what feels good"*

***Carl Sagan (1934 – 1996)***

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## Abbreviations

APC	Allophycocyanin
APC	Antigen Presenting Cell
AP-1	Activator protein 1
AR	Acute rejection
ATN	Acute Tubular Necrosis
BCC	Basal Cell Carcinoma
BMP	Bone morphogenetic protein
BSA	Bovine Serum Albumin
CAN	Chronic renal allograft nephropathy
CBA	Cytometric bead array
CBP	CREB binding protein
CD	Cluster of differentiation
CHO	Chinese Hamster Ovary
Ci	Cubitus interruptus
CMV	Cytomegalovirus
CNS	Central Nervous System
Cos-2	Costal-2
COUPTF-II	Chicken Ovalbumin Upstream Promotor Transcription Factor-II
C-Shh	Carboxy terminal form of Shh
CSE	Control standard endotoxin
C <sub>T</sub>	Threshold cycle
DAG	Diacylglycerol
DC	Dendritic Cell
Dhh	Desert Hedgehog
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
Dpp	Decapentaplegic

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Fetal Calf Serum
FFPE	Formalin-fixed, paraffin-embedded
FGF	Fibroblast growth factor
Fu	Fused
GDF	Growth and differentiation factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GRO	Melanoma growth stimulatory activity
HBSS	Hanks buffered saline solution
Hh	Hedgehog
Hip	Hedgehog interacting protein
HLA	Human Leukocyte Antigen
HRP	Horseradish peroxidase
HSP	Heat shock protein
HSPGs	Heparan sulphate proteoglycans
<sup>3</sup> H-TdR	Tritiated methyl thymidine
HUVECS	Human umbilical cord endothelial cells
IBE	Immortalised brain capillary endothelial cells
ICAM-1	Intercellular adhesion molecule-1
ICOS	Inducible co-stimulator
IFN- $\gamma$	Interferon gamma
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
Ihh	Indian Hedgehog
IKK	I $\kappa$ B kinase complex
IMDM	Iscove's Modified Dulbecco's Medium
iNOS	Inducible nitric oxide synthase
IL	Interleukin
IP <sub>3</sub>	Inositol-3,4,5-triphosphate
IP-10	IFN- $\gamma$ -inducible protein-10

IRG-1	Immune-responsive gene 1
ITAM	Immunoreceptor Tyrosine-based Activation Motif
JNK	Jun N-terminal kinase
LAL	<i>Limulus</i> Amebocyte Lysate
LDH	Lactate dehydrogenase assay
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
LRW	LAL reagent water
Mal	MyD88 adapter-like
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
MIG	Monokine induced by gamma-interferon
MIP	Macrophage inflammatory protein
MLR	Mixed leukocyte reaction
MMC	Mitomycin C
MMF	Mycophenolate mofetil
mRNA	Messenger Ribonucleic Acid
NF- $\kappa$ B	Nuclear factor $\kappa$ B
NFAT	Nuclear factor of activated T cells
N-Shh	Amino terminal form of Shh
OD	Optical density
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCT	Proximal convoluted tubule
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PEG	Polyethylene glycol
PI3K	Phosphoinositide-3-kinase
PIP <sub>2</sub>	Phosphatidylinositol-4,5-biphosphate

PKA	Protein kinase A
PLC- $\gamma$	Phospholipase C- $\gamma$
PMB	Polymyxin B
PMSF	Phenylmethanesulphonylfluoride
PRRs	Pattern Recognition Receptors
Ptc	Patched
RANTES	Regulated on activation normal T cell expressed and secreted
RAP	Receptor associated protein
REC	Renal epithelial cell
rShh	Recombinant Sonic Hedgehog
RT-PCR	Reverse transcription polymerase chain reaction
Shh	Sonic hedgehog
Smo	Smoothed
SSD	Sterol sensing domain
TCR	T cell receptor
TGF- $\beta$	Transforming Growth Factor- $\beta$
Th	T helper
TIRAP	TIR domain-containing adapter protein
TLR	Toll-like receptor
Tm	Annealing temperature
TNF $\alpha$	Tumour necrosis factor alpha
TOLLIP	Toll-interacting protein
UVB	Ultraviolet B
VCAM-1	Vascular cell adhesion molecule - 1
VEGF	Vascular Endothelial Growth Factor
Wg	Wingless

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## **Chapter 1**

### **Introduction**

## 1.1 Context

The themes underlying the work presented in this thesis have arisen as a result of several key reports investigating novel roles for developmental signalling pathways in adult physiology and pathophysiology. The Sonic hedgehog signalling pathway is one such pathway. It is known to have a role in a variety of developmental processes but, more recently, its expression has been demonstrated in adult tissues prompting speculation regarding its role in the adult. Three key publications formed the basis to the work presented in this thesis and these have been discussed in detail below.

Together, they highlight possible roles for Shh signalling in inflammation, fibrosis and peripheral immunity, roles that have, to date, been little explored. The work investigating the role of Shh signalling in inflammation and fibrosis has, as will be seen below, focussed on its possible role in the context of lung inflammation and fibrosis. Although it is well established that Shh signalling plays a role in the developing lung, more recent evidence has shown that that Shh signalling is also upregulated in the epithelium in fibrotic lung conditions in the mouse and human. This, together with recent evidence suggesting that Shh signalling components are expressed on peripheral immune cells including T cells and macrophages, and, further, that signalling through the pathway appears to modulate the proliferation and cytokine profile of CD4<sup>+</sup> T cells, suggests that Shh signalling may be important at the epithelium-immune cell interface and may play a role in linking the innate and adaptive immune systems. Indeed, Shh signalling has the potential to be involved in a number of pathologies and, specifically, highlights possible roles for Shh signalling in other conditions characterised by inflammatory cell infiltration and fibrosis.

In this regard, the kidney provides an interesting area for further study. Little is known about Shh signalling in the adult kidney although the developing kidney, like the developing lung, is influenced by hedgehog signalling. Furthermore, a number of chronic renal diseases show evidence of fibrosis. In this regard however, the transplanted kidney affected by chronic renal allograft nephropathy (CAN) is of specific interest. As discussed further below, fibrosis is one of the histopathological hallmarks of CAN and the fibrosis is associated with an inflammatory cell infiltrate



comprising macrophages and T cells. Given that Shh signalling may be upregulated in fibrotic conditions, that Shh signalling has been shown recently to be relevant to T cell effector function and that macrophages express the receptor for Shh and may thus be able to respond to a Shh signal, these factors highlight a possible role for Shh signalling at the epithelial-immune cell interface and in CAN. Like fibrotic conditions of the lung, CAN occurs even in the face of strong immunosuppressive therapy, thus highlighting the need for further exploration into the mechanisms contributing to its development and potentially presenting the Shh signalling pathway as a possible target for therapeutic intervention.

Thus, it is the possible role in CAN and contribution to peripheral immune mechanisms relevant to the epithelium-immune cell interface that the work presented in this thesis has sought to evaluate. Specifically, and based on the recent reports in the literature investigating the role of Shh signalling in lung fibrosis and peripheral immunity, this work has sought to investigate 2 central avenues of enquiry. Firstly, the possible importance of Shh signalling in the normal adult kidney and the kidney affected by CAN is explored. In the second part of the thesis, and relevant to interactions that may be occurring at the epithelium-immune cells interface, the role of Shh signalling in the effector function of macrophages and CD4<sup>+</sup> T cells has been evaluated.

In this first introductory chapter, the background leading up to the work presented in this thesis will be discussed. In the first part, general discussion of the Shh signalling pathway and the recent work concerning its roles in fibrotic lung disease and peripheral immunity will be reviewed. This will then be followed by an introduction to the poorly understood process of CAN. Given that macrophages and T cells are central to innate and adaptive immune responses and that recent reports suggest that Shh signalling may be relevant and important to the effector function of these cells, the concepts of macrophage and T cell activation are introduced in the final part of this chapter.

## 1.2 The Sonic Hedgehog Signalling Pathway

### 1.2.1 Introduction

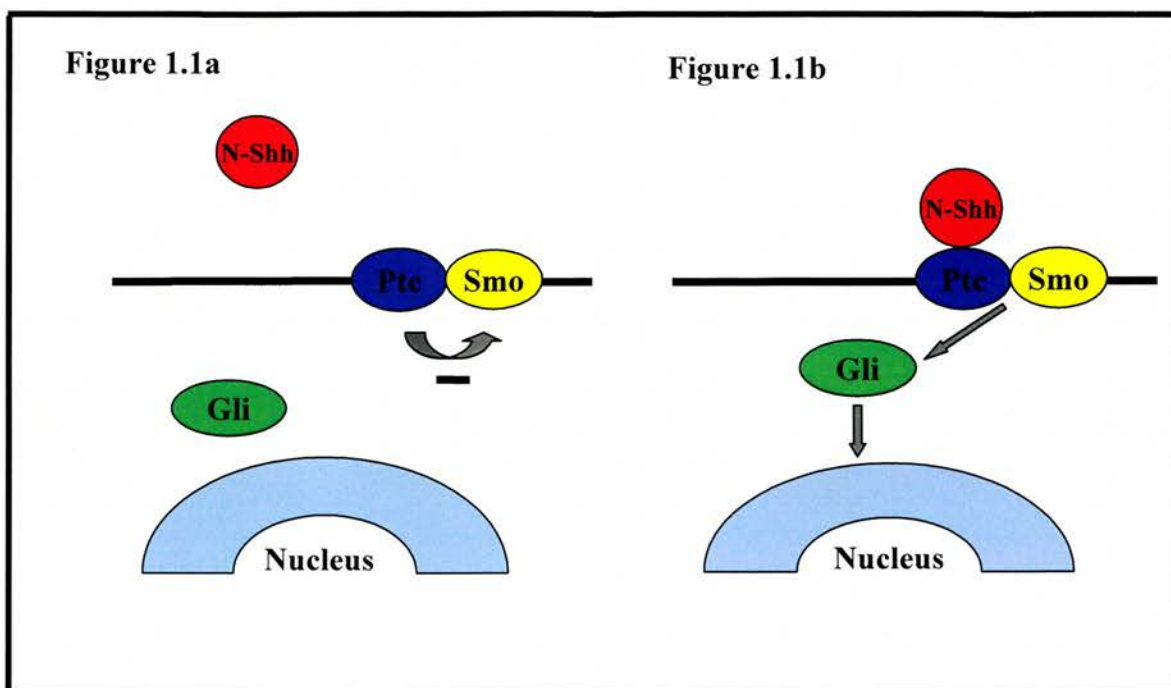
The Hedgehog (Hh) family of proteins has an important role in determining both cell fate and proliferation during development. Discovered in *Drosophila*, the hedgehog gene was initially identified during a screening process for embryonic patterning defects<sup>1</sup>. The family derives its name from a mutant form of *Drosophila* which, during its embryonic development, has prominent changes (denticles) in regions normally associated with bare cuticle. The Hh pathway is highly conserved through evolution and there are 3 vertebrate homologues of *Drosophila* Hh; Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh)<sup>2-4</sup>. All have roles during embryogenesis but, of these, Shh is the best characterised. During development, Shh signalling plays a crucial role in the regulation of embryonic patterning most notably in the neural tube, limbs, gut and lung<sup>4-7</sup>. The contribution of Ihh is most notable during the formation and growth of the limb, where it regulates the pace of chondrocyte differentiation<sup>8,9</sup>. Ectopic expression of Ihh delays hypertrophy and subsequent replacement of cartilage with bone<sup>8</sup>. Ihh also plays a role in gut organogenesis<sup>4,10</sup>, vasculogenesis and hematopoiesis<sup>11</sup>. Roles for Dhh include control of gonad and germ line development and peripheral nerve sheath formation<sup>12</sup>.

In humans, Shh is located on the distal long arm of chromosome 7 (7q36). Targeted disruption of Shh signalling results in embryonic lethality<sup>13</sup>. Mutations of the *Shh* gene result in holoprosencephaly<sup>14</sup>, a syndrome incorporating a variety of facial and cranial defects such as cyclopia. Defects in distal limb structures and the foregut are also evident<sup>15</sup>. The latter include oesophageal atresia and stenosis, tracheo-oesophageal fistula, gut malrotation, reduced smooth muscle, annular pancreas, imperforate anus and lung hypoplasia<sup>7,16</sup>. Similar features develop in animals exposed to the teratogen and Shh antagonist, cyclopamine<sup>17</sup>, and in patients with Smith-Laemli-Opitz syndrome, a disease characterised by deficient cholesterol biosynthesis<sup>18</sup>.

Aside from its role in developmental patterning, Shh also acts as a growth factor in a large number of different cell types including CNS precursors<sup>11</sup>, haematopoietic stem cells<sup>19</sup>, T cells<sup>20,21</sup> and epithelial cells<sup>22</sup>, an effect at least partly mediated via a decrease in cyclic-AMP-dependent protein kinase A (PKA) activity<sup>23</sup>. Indeed, mutations leading to the constitutive activation of the Shh signalling pathway have been linked to the development of a number of malignancies including sporadic<sup>24,25</sup> and familial<sup>26,27</sup> basal cell carcinomas, medulloblastoma<sup>28</sup> and rhabdomyosarcoma.

### 1.2.2 The Shh Signalling Pathway

The Shh signalling pathway is outlined in Figure 1.1. Shh, like its *Drosophila* counterpart, Hh, is synthesised in a 45 kDa precursor form. Autoproteolytic cleavage in the endoplasmic reticulum results in the formation of a biologically active 19kDa amino terminal form (N-Shh) and a 25kDa carboxy-terminal region (C-Shh)<sup>3,29</sup>. The signalling activity of Shh resides in the amino terminal<sup>6,30,31</sup> whereas the 25kDa carboxy-terminal region acts as a cholesterol transferase<sup>32</sup>, responsible for the covalent linking of cholesterol to the carboxy terminus of N-Shh during the autoproteolysis step<sup>29,32,33</sup>. Although it is not known why cholesterol is covalently linked to the carboxy terminus of N-Shh, it has been proposed that its function is to allow N-Shh to remain attached to the cell surface therefore promoting short-range signalling between neighbouring cells and, thus, spatially restricting the localisation of the Hh signal<sup>32,34</sup>. Furthermore, it may be that the cholesterol modification assists in localising Hh to lipid rafts within the membrane<sup>35,36</sup>, thus helping to concentrate Hh ligands, increasing the likelihood of ligand/receptor interaction and directing intracellular transport within epithelium and/or the sequestration of Shh within cells<sup>37,38</sup>. In *Drosophila*, the cholesterol modification seems to be required to target cleaved Hh to a delivery system. This system includes Dispatched, a protein similar to Ptc required for the release of Hh from the secreting cell, and Tout velu, an enzyme involved in heparan sulphate biosynthesis required for the efficient transport of, and response to, the Hh protein signal<sup>37,38</sup>. In the absence of Dispatched, the levels of Hh remains high in Hh producing cells. However, Hh is not released from cells and thus, insufficient Hh signal passes to responding cells to allow triggering of downstream pathways.



**FIGURE 1.1**

### **The Sonic Hedgehog Signalling Pathway**

Shh is synthesised in a 45 kDa precursor form. Autoproteolytic cleavage in the endoplasmic reticulum results in the formation of a biologically active 19kDa amino terminal form (N-Shh). Ptc is the receptor for Shh and binds Shh with high affinity. In the absence of Shh stimulation, Ptc represses the activity of a further protein Smoothened (Smo) and this repression of Smo by Ptc prevents the activation of the Gli family of transcription factors (Figure 1.1a). When Shh binds to Ptc, the repression by Ptc on Smo is lifted, thus allowing Smo signalling and the activation of the downstream transcriptional targets of Shh signalling through the Gli transcription factors (Figure 1.1b).

In addition to the cholesterol modification, a second lipid modification, palmitoylation of the N terminus, occurs after the cholesterol modification and may enhance the signalling activity of Shh<sup>39</sup>. Further work from *in vitro* assays suggests that neither cholesterol nor palmitoyl modification increases the affinity of Hh proteins for Ptc. However, both processes appear to increase the specific activity of the Hh protein<sup>39</sup>.

The receptor for Shh is the product of a tumour suppressor gene, *Ptc*<sup>40</sup>, originally identified as the gene responsible for Gorlin's syndrome, an autosomal dominant disorder characterised by multiple basal cell carcinomas, medulloblastoma and ovarian fibromas and other developmental anomalies<sup>27,41</sup>. The *Ptc* gene product is a transmembrane protein with 12 membrane-spanning domains and 2 extracellular loops<sup>42,43</sup>. *Ptc* is expressed in all known targets of Hh signalling<sup>27,44</sup> and binds Shh with high affinity<sup>40</sup>. In the absence of Shh stimulation, *Ptc* represses the activity of a further protein Smoothed (Smo)<sup>45,46</sup> which has marked structural similarity to the G-protein coupled receptors<sup>46</sup>. Smo is required to transduce the Shh signal<sup>45-47</sup> and, in *Drosophila*, is required for all Hh signalling<sup>48</sup>. In the absence of a Shh signal, the repression of Smo by *Ptc* prevents activation of the downstream targets of Shh signalling<sup>49</sup>. Together, *Ptc* and Smo form a receiving unit for Shh<sup>27,40</sup> although it appears that only *Ptc* is capable of binding to Shh, since, in the absence of *Ptc*, Shh fails to bind Smo<sup>40,50</sup>. When Shh binds to *Ptc*, the repression by *Ptc* on Smo is lifted, thus allowing Smo signalling and the activation of the downstream transcriptional targets of Shh signalling. In mice lacking *Ptc* activity, the Hh signalling pathway response genes are constitutively activated in target tissues by the permanent activation of Smo<sup>11</sup>. This results in a neoplastic state characterised by proliferating, undifferentiated cell populations<sup>27,41</sup>. Activating mutations in *Smo* and *Shh* have been shown to result in similar phenotypes to those seen when *Ptc* expression is absent<sup>24,51</sup>.

The exact mechanism by which *Ptc* exerts its effects on Smo has yet to be determined although it has been proposed that the inhibition of Smo by *Ptc* requires them to be in close proximity<sup>52</sup>. Early studies suggested a simple stoichiometric

regulation of Smo by Ptc. Indeed, initial cell culture experiments based on overexpression experiments proposed that Ptc and Smo may interact directly to form a complex<sup>40,45</sup>. The experiments further suggested that when Hh binds to Ptc, the complex undergoes conformational change resulting in the activation of Smo. However, other studies investigating a possible stoichiometric relationship have not been supportive of such an interaction<sup>53</sup>. Experiments in *Drosophila* have revealed that Ptc and Hh colocalise to intracellular vesicles in Hh-responding cells<sup>37</sup>. Other studies in mammalian tissue culture studies have shown that soluble recombinant Shh protein can be internalised by cells transfected with *Ptc*<sup>54</sup>. Some authors posit that both Ptc and Hh are targeted to the lysosome<sup>55</sup>, at least in some cells. Others have proposed that the internalisation process is dynamin-dependent<sup>54</sup> suggesting that internalisation is mediated via clathrin-coated pits<sup>54</sup>. Some evidence, however, lends support for a role for caveolae in the internalisation process. Indeed, Ptc has been shown *in vitro* to associate with caveolin-1<sup>56</sup>. Other authors though do not concur with a role for caveolin in Ptc internalisation, viewing it instead as a means of delivering Ptc to the plasma membrane<sup>56</sup>. Lipid rafts have also been implicated in both Ptc internalisation and the interaction of Ptc with Smo. Indeed, Hh has been demonstrated to accumulate in lipid rafts<sup>35</sup>. Smo also localises to lipid rafts but whether the association of Ptc and Smo occurs before or after lipid raft integration is not yet clear<sup>57</sup>.

Other insights into the nature of the relationship between Smo and Ptc have come from studies in *Drosophila* lending support to more indirect models of Smo regulation by Ptc. Indeed, the findings from a number of studies have suggested that, although Hh and Ptc colocalise in Hh-responding cells, Smo and Ptc do not colocalise in responding cells<sup>58,59</sup>, thus supporting a more indirect interaction between Ptc and Smo. Indirect models proposed include a catalytic regulation by Ptc and evidence in favour of this comes from studies demonstrating a 45:1 ratio of Smo:Ptc resulting in 80% reduction in Smo activity<sup>53</sup>. Other studies have posited that Ptc may act by regulating Smo trafficking within the cell<sup>60,61</sup>. Indeed, Smo downregulation appears to be dependent on the Ptc sterol sensing domain (SSD)<sup>60,61</sup> supporting a role for Ptc in the trafficking of Smo through a membrane



compartment<sup>11</sup>. Other proposed mechanisms of inhibition include the possibility that Ptc might control Smo function by influencing its interaction with cellular small molecules<sup>62</sup>.

### 1.2.3 Cyclopamine

Cyclopamine is a plant steroid alkaloid derived from *Veratrum californicum*. Ewes that consume this plant give birth to lambs with features of holoprosencephaly<sup>63</sup> and the administration of cyclopamine to vertebrate embryos produces a phenotype consistent with loss of Hh signalling<sup>15</sup>. In NIH-3T3 cells transiently transfected with both luciferase reporter and Smo complementary DNA, overexpression of Smo induced reporter expression 10-fold, an effect suppressed by 5-20 $\mu$ M cyclopamine, providing *in vitro* evidence for an inhibitory effect of cyclopamine on Smo activation and, thus, Shh signalling. Since the discovery that cyclopamine inhibits Shh signalling, it has been used extensively in both *in vitro* and *in vivo* experiments to abrogate Shh signalling<sup>64-68</sup>. Although the precise mode of action of cyclopamine has not been determined, cyclopamine appears to inhibit the cellular response to the Hh signal by inhibiting activated Smo in a Shh and Ptc-independent manner<sup>17,64,69</sup>, possibly through binding directly to the heptahelical domain of Smo<sup>70</sup>. The activation status of Smo appears to be critical in determining whether cyclopamine will inhibit Hh pathway activation as evidenced by experiments demonstrating little effect of cyclopamine on the reporter expression induced by the oncogenic Smo mutants W539L<sup>24</sup> and S537<sup>25</sup>. In the former study, all activating Smo mutants were cyclopamine resistant, the level of resistance correlating positively with increased potency, consistent with a model in which cyclopamine affects the balance between activity states of Smo<sup>64</sup>.

### 1.2.4 The Gli family of transcription factors

In *Drosophila*, transcription in the Hh signalling pathway is mediated by the zinc finger transcription factor Cubitus interruptus (Ci), a protein with 3 key functional domains. These domains include an amino-terminal region involved in transcriptional repression<sup>71</sup>, a zinc finger domain that binds to DNA<sup>72</sup> and a carboxy-

terminus that mediates activation of transcription<sup>71</sup>. Ci forms a complex in the cytoplasm of the cell with 2 other proteins, Fused (Fu) and Costal-2 (Cos-2). Fu is a serine-threonine kinase and positive mediator of the hedgehog signal whereas Cos-2 acts to inhibit its effects<sup>73</sup>. Cos-2 is responsible for the binding of the complex to microtubules<sup>74</sup>. Proteolysis of the Ci gives rise to an amino-terminal containing region (Ci75) and a carboxy terminus. Ci-75 contains the zinc finger domain, which migrates to the nucleus and represses hedgehog target gene transcription<sup>74</sup>. Hh signalling in *Drosophila* inhibits cleavage of full-length Ci and, thus, the Ci-Fu-Cos-2 complex dissociates from the tubules<sup>75</sup>. Conversely, activated Ci binds to the transcriptional coactivator, CREB-binding protein (CBP), allowing the expression of Hh target genes<sup>76</sup>.

In vertebrates, there are three known homologues of Ci constituting the Gli family of transcription factors<sup>77,78</sup>. The roles of the individual Gli family members have yet to be fully delineated, a process hampered by the partial redundancy and overlapping domains of Gli expression. Nevertheless, results from a number of different studies have shown that different Gli members appear to be responsible for different activator and repressor functions<sup>23</sup>.

*Gli1* was initially identified as a potential oncogene in human glioblastoma<sup>79</sup>. Subsequently, the family of vertebrate *Gli* genes that includes *Gli1*, 2 and 3 was cloned and found to be expressed in many organs during development<sup>80</sup>. Of the Gli transcription factors, Gli1 appears to act solely as an activator inducing the upregulation of target genes<sup>81</sup> and functioning in a similar manner to Ci<sup>78</sup>. In tissue culture cells or transgenic embryos, overexpression of *Gli1* can induce the transcription of Hh target genes in the absence of Hh activity<sup>23,82</sup>. Overexpression of Gli1 also contributes to the transformation of primary cells<sup>83</sup> and occurs in glioblastomas<sup>79</sup>, basal cell carcinomas (BCCs)<sup>84</sup>, osteosarcomas, rhabdomyosarcomas and B cell lymphomas<sup>85,86</sup>. Given these effects, it is interesting to know that mice that lack Gli1 are phenotypically normal<sup>87</sup>.



In contrast to the activating functions of Gli1, Gli 2 and 3 have both activating and repressive domains<sup>88</sup>. Gli2 plays a critical role in neural, lung and skeletal development<sup>89,90</sup> and mice with mutations in *Gli2* die at birth and exhibit a variety of developmental defects including abnormal floorplate, skeletal and lung development<sup>91</sup>. *In vivo* evidence for an activating role of Gli2 comes from mouse mutants lacking the zinc finger regions<sup>89</sup> and in transgenic flies and frog embryos, Gli2 is a weak activator<sup>81</sup>. Deletion of the N-terminal domain of the protein, however, turns Gli 2 into a strong activator, capable of inducing normally Shh-dependent cell types<sup>88</sup>.

Although capable of acting as both an activator and a repressor, the main role for Gli3 appears to be as a repressor of Hh targets as well as of Shh itself<sup>92</sup>. This repression is mediated by proteolytic cleavage of full-length Gli3 into a repressor form. The Gli3 mutant phenotype lends further support for a repressive role for Gli3. Loss of Gli3 results in dorsal brain defects and limb polydactyly that are associated with ectopic activation of the Shh pathway and human mutations in the *Gli3* gene cause Greig syndrome, a condition characterised by syndactyly and facial anomalies<sup>93</sup>, and Pallister-Hall syndrome, an autosomal dominant disease incorporating a spectrum of defects such as polydactyly, syndactyly, imperforate anus, anteverted nares and other facial abnormalities<sup>94</sup>. Shh signalling functions to inhibit the formation of the repressor form of Gli3<sup>95</sup> with removal of Gli3 function in Shh mutants largely rescuing the Shh mutant defects demonstrating that, at least in part, the Shh mutant phenotype is due to an excess of Gli3 repressor<sup>96</sup>.

### 1.2.5 Targets of Hh signalling

Hh signalling activates a number of target genes. Somewhat paradoxically, one of the best recognised downstream targets of Hh signalling is Ptc itself<sup>97</sup>, with high levels of Ptc expression commonly used to indicate a transcriptionally active Hh signalling pathway<sup>98-100</sup>. It has been proposed that this upregulated Ptc expression in response to Shh signalling serves to sequester Hh thereby limiting its action<sup>101</sup> and, further, that this negative regulatory mechanism may help to generate a Hh concentration

gradient<sup>101</sup>. Gli1 is also a transcriptional target of Hh signalling and, like Ptc, is often used to indicate a transcriptionally active Hh signalling pathway.

A number of other pathways appear to be activated by Hh signalling. Notably, in the context of this thesis, these pathways include growth factors such as members of the TGF- $\beta$  superfamily, vascular endothelial growth factor (VEGF)<sup>102,103</sup> and some of the fibroblast growth factors<sup>104</sup>. Other targets of Hh signalling include members of the *Hox* gene family, the transcription factor N-myc, cyclin D2, insulin-like growth factor binding proteins, Deltex 1, osteopontin and plakoglobin<sup>105,106</sup>. Of these downstream targets of Hh signalling, the growth factors TGF- $\beta$  and VEGF are of particular interest since they are directly relevant to avenues pursued during the course of this thesis.

The TGF- $\beta$  superfamily is a large family of secreted growth factors that affect many developmental processes including growth and differentiation<sup>107-109</sup>. Members of the family act downstream of Hh proteins in several developmental systems<sup>110-113</sup>. In *Drosophila*, Hh signalling controls the expression of the long range morphogen and TGF- $\beta$  superfamily member, Decapentaplegic (Dpp)<sup>114</sup>, and it is believed that activation of Dpp allows Hh to control patterning events in the imaginal discs<sup>110,115,116</sup>. Hh signalling also controls the expression of *Wingless*<sup>57,72</sup>, another morphogen family which interacts with *Dpp* to regulate both cell fate and proliferation during development<sup>117</sup>. In vertebrates, the TGF- $\beta$  superfamily includes the 3 vertebrate isoforms of TGF- $\beta$ , the activins and inhibins, growth and differentiation factors (GDFs) and the bone morphogenetic proteins (BMPs)<sup>118</sup>. In vertebrates, the closest homologues to *Drosophila* Dpp are the BMPs, BMP-2 and BMP-4<sup>119</sup>. These 20-30kDa homodimeric proteins regulate many morphogenetic events during embryonic development, playing an integral role in epithelial-mesenchymal interactions<sup>119</sup> and, as their name would suggest, functioning primarily to induce chondrocytic and osteoblastic differentiation from mesenchymal progenitors. Both BMP-2 and 4 have been shown to be regulated by Hh signalling<sup>111</sup>. Shh induces ectopic expression of BMP-2 when misexpressed in the chick limb<sup>111</sup> and Hh signalling appears to act upstream of BMP-4 signalling to induce the

proliferation of primitive human blood cells<sup>19</sup>. *Dynamo*, a novel zebrafish member of the TGF- $\beta$  superfamily is also upregulated by Shh<sup>120</sup>. Interestingly, although members of the TGF- $\beta$  superfamily appear to be downstream targets of Hh signalling, a direct association between Hh signalling and the regulation of any one of the 3 isoforms of TGF- $\beta$ , has not been demonstrated. The same, however, may not be true of the angiogenic factor and endothelial cell mitogen, VEGF. This growth factor has been shown in a number of recently published studies to be a downstream target of Hh signalling. Indeed, a direct association between Hh signalling and VEGF has been proposed, a relationship that is discussed in Section 1.2.9 in more detail.

### 1.2.6 Other players in the Shh pathway

In addition to the Shh-Ptc axis described above, there is a growing body of evidence to suggest that some effects attributed to Hh signalling may be mediated by a pathway outwith the Shh-Ptc pathway described above<sup>121-123</sup>. Indeed, other receptors have been reported to bind Shh. These include Ptc2, Hedgehog interacting protein (Hip)<sup>124</sup>, vitronectin<sup>125</sup> and megalin<sup>126,127</sup>.

The *Ptc2* gene was identified in 1998<sup>128,129</sup>. It shares 60% amino acid identity with Ptc1 and has some characteristics similar to Ptc such as the extracellular loops that mediate the interaction with Hh<sup>130</sup>. Furthermore, Ptc2 associates with Smo<sup>128</sup>. Nevertheless, the specific role of Ptc 2 in the Hh pathway remains unclear. It appears to be coexpressed with Shh-producing cells and its transcription, unlike that of Ptc, is independent of Hh signalling<sup>131</sup>. Indeed, the expression of Ptc2 is modulated by, but not dependent on, Hh signalling. Furthermore, Ptc2 is not able to replace Ptc function in tumour cells<sup>130</sup> and during development in the mouse<sup>40</sup>, suggesting that Ptc and Ptc2 have distinct and different functions.

Hip is a membrane-associated glycoprotein<sup>124</sup> that binds to all 3 vertebrate homologues of Hh with a similar affinity to that of Ptc. As with Ptc-expressing cells, Hip-expressing cells are located next to those expressing Hh<sup>132</sup>. *Hip*, like *Ptc* and *Gli1*, is a transcriptional target of hedgehog signalling, induced by ectopic Hh signalling and lost in Hh mutants<sup>124</sup>. The exact function of Hip remains to be fully

elucidated. Interestingly, a Hip orthologue in *Drosophila* has not yet been identified<sup>124</sup> and Hip remains relatively poorly characterised. It appears to function in a negative manner to attenuate a Hh signal since overexpression of Hip attenuates Hh signalling in mice<sup>132</sup>. It remains to be seen whether downstream targets such as Gli family members are directly modulated by Hip interactions. It may be that Hip mediates effects outwith those of the relatively well-characterised Shh signalling pathway, a possibility highlighted recently by the work of Testaz et al<sup>121</sup>.

Megalin, also known as gp330, is a 600kDa transmembrane protein belonging to the Low Density Lipoprotein (LDL) receptor superfamily<sup>133,134</sup>. Megalin is expressed in many epithelial cells including the proximal tubular cells and glomerular podocytes of the kidney, type II pneumocytes, small intestinal epithelium, the visceral yolk sac and the cytotrophoblast of the placenta, thyroid and parathyroid cells, endometrium, oviduct, epididymis, ependymal cells, labyrinthine cells of the inner ear and the ciliary epithelial cells of the eye<sup>135</sup>. Megalin requires the chaperone protein, receptor-associated protein (RAP), in order to function optimally<sup>136</sup>. Although the significance of the interaction is not entirely clear, a recent study has demonstrated that Shh is a novel ligand for megalin<sup>126</sup>. Interestingly, in animals rendered deficient in megalin, holoprosencephaly is also seen, lending support for megalin being a possible receptor for Shh. Megalin-expressing cells were shown to internalise Shh through a mechanism that could be inhibited by antagonists of megalin, namely anti-RAP and anti-megalin antibodies<sup>126</sup>. Interestingly, in the same study, when chloroquine was used to inhibit lysosomal proteinase activity, megalin-mediated N-Shh endocytosis did not target Shh to the lysosomes, one of the mechanisms thought to be involved in Ptc-mediated Shh degradation.

### **1.2.7 Shh signalling in the kidney**

As outlined above, Shh signalling is integral to the development of a number of organ systems. The developing kidney is no exception. Mice deficient in Shh develop a solitary kidney<sup>137</sup> probably as a secondary consequence of the midline defects occurring in the early mutant embryos. Deletions in the chromosome at the

site containing Shh are associated with defects such as hydroureter, hydronephrosis and renal hypoplasia<sup>138</sup>. Furthermore, Shh expression has been demonstrated in the urothelium of the developing kidney<sup>4,138,139</sup>. In these studies, Shh expression was restricted to the distal collecting ducts generated from the first branches of the ureteric bud and the early E11.5 ureter, the findings further confirmed in other studies using conditional loss of function models<sup>138</sup>. Interestingly, high levels of Ptc expression were observed in the mesenchymal cells adjacent to the Shh-expressing epithelium of the collecting ducts and the ureter. At birth, Shh was shown to be expressed in the inner medullary collecting ducts, the renal pelvic epithelium and the ureteric epithelium, with concurrent Ptc expression in the mesenchyme of the medulla and renal pelvis consistent with the possibility that Shh may serve as a mitogen to promote proliferation of ureteral mesenchymal cells.

Aside from Shh itself, mutations in other components of the Shh signalling pathway can give rise to renal defects. Gli2<sup>-/-</sup>;Gli3<sup>+/-</sup> mice develop a horseshoe kidney<sup>137</sup> and, in humans, mutations in the Shh signalling pathway have been linked to the renal anomalies associated with the VACTERL syndrome<sup>137</sup>, a disorder characterised by vertebral defects, anal atresia, tracheoesophageal fistula, radial and renal dysplasia, and limb abnormalities. Other downstream elements of the Hh signalling cascade have also been implicated in renal development. For example, the recognised targets of Hh signalling, BMPs 2 and 4 are also expressed in the developing kidney as are mRNA transcripts for a number of other BMPs, including *BMPs 3,5,6* and 7 which have been found to be expressed in the ureter, developing tubules, glomeruli and metanephric mesenchyme throughout metanephric development<sup>140</sup>. Determining the precise role for BMPs in renal development, however, is difficult. Indeed, for example, BMP2 null mice die early and heterozygotes fail to display any obvious renal phenotype<sup>141</sup> thus making evaluation of the precise role for BMP2 difficult to determine. BMP2 along with BMP7 is proposed to play a prominent role in the regulation of branching morphogenesis in the developing kidney<sup>140,142</sup>. In embryonic kidney explants and the IMCD-3 model of collecting duct morphogenesis, the addition of recombinant BMP2 and BMP7 appears to regulate branching morphogenesis<sup>142</sup>. BMP4 is expressed in the stromal cells around the Wolffian duct

and stalk of the ureteric bud<sup>143</sup> and may regulate ureteric bud formation. In explanted embryonic metanephroi, the administration of BMP4 reduces ureteric epithelial branch formation and disrupts mesenchymal differentiation<sup>144</sup>. BMP4 may also be involved in determining the anterior-posterior axis of the kidney<sup>145</sup>.

Aside from Shh, other Hh proteins appear to be relevant in renal development. For example, Indian hedgehog signalling also appears to play a role in the developing kidney where epithelial expression in differentiated tubular epithelium may serve to signal to adjacent proliferating cells and maintain their undifferentiated state. It is first detected in the murine metanephros at E14.5. By birth, Ihh is expressed in the epithelium of the outer medulla and cortex<sup>146</sup> with no expression in the collecting ducts or ureter. Gene expression then increases with age, and is maximally expressed in the adult<sup>146</sup> where expression is observed in the proximal tubules.

### **1.2.8 Shh signalling and the immune system**

That Shh signalling may play a role in the immune system has been suggested by several lines of evidence. In 2000, Outram et al first demonstrated the presence of components of the Hh cascade in the murine thymus and their involvement in T cell maturation<sup>147</sup>. Extracts of whole murine thymus were shown to express mRNAs for *Shh*, *Ptc1*, *Ptc2*, *Smo*, *Gli1*, *Gli2* and *Gli3* and for the target of Hh signalling, *BMP-4*<sup>147</sup>. Subsequent sorting of thymic cell populations demonstrated the expression of *Ptc* and *Smo*, but not *Shh*, by thymocytes and the expression of *Shh* by thymic epithelium, leading the authors to speculate that thymocytes may be able to respond to, but not generate, a Shh signal<sup>147</sup>. In a further report by the same group, immunohistochemistry studies confirmed the protein expression of Shh by the epithelial cells of the thymus<sup>148</sup> and 85% of the thymocytes were found to express Ptc1 protein. Conversely, 35% of the thymocytes expressed Smo protein with the most concentrated areas of expression corresponding to clusters of cells mixed with thymic epithelial cells suggesting that Smo expression was highest in areas where Hh signalling pathway components were in close proximity for signalling to occur<sup>148</sup>. Only early T-cell progenitors and thymic epithelium were found to express the Gli



genes *Gli1*, 2 and 3 and these genes were not expressed in double positive and CD4+ thymocyte populations<sup>148</sup>, a finding supported by the findings of Bhardwaj et al who demonstrated the expression of *Ptc1* and *Smo*, but not any of the *Gli* genes in human peripheral CD3+ and CD19+ lymphocytes<sup>19</sup>.

That active Hh signalling may occur in the thymus and influence early thymocyte development was also demonstrated by Outram et al<sup>147</sup>. Culturing of thymocytes in the presence of the anti-Hh antibody, 5E1 was shown to accelerate the progression of double negative into double positive thymocytes<sup>147</sup>. Furthermore, culturing the thymocytes in the presence of Shh arrested thymocyte differentiation at the CD25+ DN stage after TCR $\beta$  rearrangement. Subsequently, Bhardwaj et al<sup>19</sup> provided further evidence to suggest that Shh signalling may be important in regulating the proliferation and differentiation of haematopoietic precursor cells. Treatment of highly purified primitive blood cells (CD34<sup>+</sup> CD38<sup>-</sup>Lin<sup>-</sup>) cells with exogenous Shh induced the expansion of these cells whilst neutralisation of Shh signalling inhibited cellular proliferation, maintaining the progenitors instead in an undifferentiated state. Bhardwaj et al<sup>19</sup> also demonstrated that the effect on proliferation is, at least in part, mediated via BMP-4 since Noggin, a BMP-4 antagonist, was able to inhibit both Shh-induced and BMP-4-induced proliferation of progenitor cells<sup>19</sup>.

In addition to the above roles proposed for Shh signalling in T cell development, 2 recent publications have explored the possible role for Shh signalling in peripheral T cells in the adult. The studies from Edinburgh investigated the expression of Shh signalling components and the role of Shh signalling in adult CD4+ T cells. In contrast to the earlier findings in thymocytes in which *Shh*, *Gli1*, *Gli2* and *Gli3* were not expressed by thymocytes, depletion-purified CD4+ T cells were found to express *Shh*, *Ptc* and *Gli1* in murine and human *in vitro* experiments<sup>20,21</sup>. Lowrey et al<sup>21</sup> further demonstrated that the addition of exogenous recombinant Shh (rShh) enhanced the proliferation of anti-CD3/antiCD28 activated murine CD4+ T cells, an effect mediated via enhanced entry into the S/G2 proliferative phase of the cell cycle. The findings in human CD4+ T cells<sup>20</sup> were broadly in keeping with those in murine CD4+ T cells<sup>21</sup>, although different activating conditions were used in the studies and

enhanced proliferation in response to Shh was only seen in 50% human donors studied. Both groups also reported on the abrogation of the proliferative response of activated CD4<sup>+</sup> T cells when endogenous Shh signalling was blocked using the monoclonal antibody, 5E1. In the human study, this abrogation of proliferation was observed in only 50% donors studied. Stewart et al<sup>20</sup> further investigated the effect of both exogenous Shh administration and blockade of endogenous Shh signalling on activated human CD4<sup>+</sup> T cells. In these experiments, IFN- $\gamma$ , IL-2, IL-10 and IL-5 were shown to be upregulated by exogenous Shh administration, an effect observed only in the 50% donors who demonstrated changes in proliferation. Furthermore, blockade of endogenous Shh signalling resulted in the differential abrogation of cytokine production by activated CD4<sup>+</sup> T cells, with IFN- $\gamma$  and IL-2, but not IL-10 and IL-5, production being reduced by 5E1 treatment, an effect once again observed only in those donors where an abrogation in proliferation was observed in response to 5E1.

Much of the work to date investigating Hh signalling in the immune system has focussed on development and, in this regard, T cell development has attracted the most attention. Only more recently has interest focused on the adult peripheral immune system with the publication of the 2 reports on adult CD4<sup>+</sup> T cells outlined above. Indeed, very little is known regarding the expression of Hh signalling components in other adult immune cells. Of interest in this regard are the recent findings of Stewart et al<sup>149</sup> in which alveolar macrophages were shown to express Ptc protein thus providing a possible mechanism through which T cells and macrophages may be able to interact via the Shh signalling pathway.

### **1.2.9 Inflammation and repair**

That Shh is important in the cell fate decisions and early developmental patterning in the embryo is now well recognised. Much less is known about the possible role for Hh signalling in the adult. Hh signalling pathway component expression is observed in a number of organ systems in the adult, albeit at lower levels than those seen in the embryo (Reviewed by McMahon et al<sup>5</sup>). The relevance of the expression of pathway



components is, however, less clear. For certain adult processes, the role for Hh signalling appears to have been clearly established. For example, Hh signalling has been shown to be critical for hair regeneration in postnatal mice<sup>150</sup>. In other systems, the role for Hh signalling is far from clear. It has been speculated that the role of the developmental signalling pathways in the adult might be to recapitulate embryonic processes when required<sup>151</sup>. Such recapitulation of embryonic process might be called upon under conditions of damage, inflammation and repair where rudimentary reparatory processes are often very similar in nature to those occurring during embryonic development.

Interestingly in this regard, there are several lines of evidence to suggest that Hh signalling may be reactivated or upregulated at sites of damage or injury and examples of such upregulation can be found in a wide variety of organ systems (Reviewed in Fitch et al, Appendix 6). For example, Ihh signalling is recognised to be important during embryonic limb formation and growth where it regulates chondrocyte differentiation<sup>4,8</sup>. In the adult, Ihh expression has been demonstrated to be upregulated at bone fracture sites<sup>152-154</sup> and *Ihh* mRNA is detected in mature and hypertrophic chondrocytes of the fracture callus lending several authors to posit that Ihh may play a role in fracture repair<sup>152</sup>. Furthermore, cyclical mechanical stress greatly induced the expression of Ihh by chondrocytes suggesting that the *Ihh* gene is mechanoresponsive<sup>155</sup>, and may thus play a role, not only in growth, but also repair processes. Shh or Ihh may also directly modulate osteoblast behaviour since the administration of recombinant Shh, commonly used in *in vitro* studies in place of Ihh, to primary osteoblast cultures results in the formation of mineralised bone nodules, accelerating the expression and activity of alkaline phosphatase<sup>156</sup>. These results indicate that rShh may induce osteoprogenitor cells to differentiate and are consistent with further reports to suggest that Hh may induce undifferentiated mesodermal cells to form bone *in vivo*<sup>157,158</sup>.

In the adult gastrointestinal tract, Hh signalling plays a number of roles with relevance to inflammation and repair. Proliferation of the gut progenitor cells occurs in response to injury<sup>7</sup> and Shh may play a role in determining the cell fate of such

progenitor cells during the repair process. In the *Xenopus* stomach, Shh expression closely correlates with cell proliferation and the development of the adult-type epithelial phenotype<sup>159</sup>. Other authors have also suggested that Shh expression may be required in the adult to maintain the structure and function of the gastric glands. Indeed, Van den Brink et al<sup>67</sup> posited that Shh may represent a fundic gland specific soluble morphogen, loss of which may contribute to intestinal transformation and the development of malignancy. Furthermore, that injury may be linked with reactivation of the Shh signalling pathway has been suggested by the work of Dimmler et al<sup>160</sup> in studies demonstrating that upregulated Shh transcription occurs in one (23132) of three (23132, HGT-1 and AGS) gastric epithelial cell lines in response to reduced pH. It remains to be seen however whether such activation of the Hh signalling pathway in response to such injurious process occurs *in vivo*.

In experimental models in the lung, naphthalene can be used to deplete Clara cells. This depletion is followed by the proliferation of progenitor cells and the reestablishment of an intact epithelial barrier. Watkins et al<sup>161</sup> demonstrated the upregulation of Shh and Gli expression in response to this injury, with the peak in expression occurring at the same time as the re-establishment of epithelial continuity. Coincident with this upregulation an increase in the number of pulmonary neuroepithelial cells was demonstrated, a finding of relevance given the recent hypothesis that neuroepithelial cells may represent epithelial progenitor cells<sup>162</sup>. Aside from the upregulated Shh expression seen in models of acute lung injury<sup>161</sup>, upregulated Shh expression has also been demonstrated in chronic lung disease. Stewart et al<sup>149</sup> recently reported on the upregulation of Shh expression in areas of remodelling epithelia overlying fibrosis both in the human condition idiopathic pulmonary fibrosis and in an irritant-induced murine model of fibrosis. At these sites, TGF- $\beta$  and Shh expression appeared to colocalise, lending the authors to speculate that, in the damaged lung, the two pathways may communicate.

The process of angiogenesis assumes a key role in the inflammatory process. Shh signalling has been speculated to play an important role in angiogenesis as evidenced by the results from a number of recent studies<sup>65,102,103</sup>. In 2001, Pola et al reported on

the upregulation of Ptc by Shh in the coronary arteries and aortas of mice<sup>102</sup>. In the same study, using a hind-limb ischaemia model and a corneal angiogenesis model, Shh was shown to upregulate neovascularisation and blood flow, an effect mediated through increases in the number and diameter of new vessels formed. This effect occurred in association with upregulated expression of VEGF and the angiopoietins Ang1 and Ang2. Interestingly, in a further limb to the study, the administration of Shh was shown to elicit different effects to those induced by VEGF administration. In the corneal angiogenesis model, although both Shh and VEGF induced angiogenesis, Shh induced long tortuous vessels of larger calibre compared to the fine network of vessels induced in response to VEGF. Furthermore, in the hindlimb ischaemia model, Shh, but not VEGF, effected capillary ingrowth. In a subsequent paper by the same group, Shh and Ptc mRNA and protein expression were shown to be increased in the first week following injury in the hind-limb ischaemia model<sup>103</sup>. As in the earlier study<sup>102</sup>, the cells responsible for this upregulation were the mesenchymal-derived fibroblasts and it was further noted that the pattern of expression colocalised with the expression of VEGF. Interestingly, VEGF was upregulated in the ischaemic limb but this upregulation could be abrogated using the 5E1 antibody to block Hh signalling. This corroborates with the earlier reports of Lawson et al<sup>163</sup> which suggested that Shh acts upstream of VEGF and that Hh pathway activation may be a prerequisite for the postnatal angiogenic response to muscle ischaemia.

The mechanism of VEGF upregulation in response to Shh remains to be elucidated. Gli response elements are absent from the VEGF promotor region suggesting that the upregulation induced by Shh is either indirect or mediated via a Gli-independent mechanism. Gli-dependent and independent components of Shh-induced capillary morphogenesis have also been proposed by Kanda et al<sup>65</sup>. Using recombinant Shh administration to either human umbilical cord endothelial cells (HUVECs) or immortalised brain capillary endothelial cells (IBE), this group established that Shh could induce capillary morphogenesis. The authors failed to show a direct effect of Shh on the proliferation or VEGF production of endothelial cells and the effect on capillary morphogenesis appeared to be dependent on Shh-induced phosphoinositide-

3-kinase (PI3K) activity although, interestingly, independent of Gli1 nuclear translocation, a commonly used marker of Shh pathway activity. Whether, then, this Gli-independent effect occurs through a different mediator such as the orphan nuclear receptor, Chicken Ovalbumin Upstream Promotor Transcription Factor-II (COUP-2), a Gli-independent downstream target of Hh signalling<sup>164</sup> which also has a role in the development of the primary vascular plexus<sup>165</sup>, has been posited<sup>103</sup>, but remains to be established.

## 1.3 Chronic Renal Allograft Nephropathy (CAN)

### 1.3.1 Introduction

For patients with end-stage renal disease, irrespective of aetiology, renal transplantation is the treatment of choice. Following the first human renal allograft transplant performed in Russia in the 1930s, knowledge regarding renal transplantation and transplantation immunology has rapidly evolved. Although it was soon recognised that compatibility between blood and tissue components was important for successful transplantation, it was not until 1954 that the significance of this was demonstrated when the first successful kidney transplant between identical twins was performed. Some five decades later, overcoming tissue barriers between donor and recipient remains a major obstacle to successful transplantation.

The introduction of ciclosporin in the 1980s had a significant impact on the early prognosis of renal allografts and the subsequent development of newer agents and improved regimes has resulted in 1-year graft survival rates exceeding 80 and 90-95% for cadaveric and living related donors respectively<sup>166,167</sup>. Nevertheless, despite the reduction in the number and severity of acute rejection episodes, the long-term outcome of renal allografts has remained relatively constant<sup>168-170</sup>. Indeed, it is notable that the long-term graft survival remains similar to that seen in the era prior to the introduction of ciclosporin, with a predicted graft life of 7-8 years, irrespective of the source of the allograft<sup>167,169,171</sup>. There is further enthusiasm for newer immunosuppressive agents<sup>172</sup> such as tacrolimus, mycophenolate mofetil (MMF) and rapamycin but it remains to be seen whether these will have any significant impact on the long-term outcome of renal transplants.

Chronic allograft nephropathy (CAN) is a significant cause of late graft loss following renal allograft transplantation<sup>173-175</sup>. CAN is a poorly understood process that occurs independently of immunosuppressive therapy and is not amenable to treatment with conventional anti-rejection therapies. Clinically, CAN is manifested by a progressive functional deterioration in allograft function. This is associated with

a fall in creatinine clearance and an associated rise in serum creatinine secondary to a decline in glomerular filtration rate<sup>176</sup>. In most instances, proteinuria occurs<sup>174</sup> and hypertension is common. Early diagnosis of CAN is difficult since the clinical sequelae of the condition usually occur some time after the histopathological changes. Indeed, in prospective studies, mild histological changes in the graft attributable to CAN could be demonstrated in 40-76% of biopsies of kidneys with normal or near normal function<sup>173,177</sup>.

### **1.3.2 Aetiology**

Chronic allograft nephropathy is a relatively new term for the changes occurring in the failing allograft. Chronic rejection was initially the term used to describe the changes reflecting the belief that a continuous immunological insult was responsible for the pathology occurring in the chronically failing allograft. However, it is now recognised that true chronic rejection represents only one possible cause for the histopathological changes observed and CAN better describes what is likely to be a multifactorial process<sup>172,178,179</sup>. Indeed, factors which may contribute to the development of CAN include humoral<sup>180,181</sup> and cellular immune reactions and a number of alloantigen-independent factors<sup>182,183</sup>, all of which can contribute to the histopathological changes seen in the chronically failing allograft.

That immune mechanisms are important in the development of CAN is suggested by 2 key lines of evidence. Firstly, the number and/or intensity of acute rejection episodes has been shown in a number of studies to correlate strongly with the later development of CAN<sup>184-187</sup>. Furthermore in this regard, early acute rejection episodes occurring within the first 3 months post-transplantation have been shown to be less predictive of CAN than those occurring at a later stage<sup>187,188</sup>, and recurrent episodes are more often associated with the development of CAN than single episodes of acute rejection<sup>189-191</sup>. The nature of the acute rejection episode may also be important since severe episodes, those incompletely reversed with anti-rejection agents or episodes with a predominant vascular component appear to be more predictive of subsequent chronic changes<sup>188,192</sup>.



The second line of evidence supporting an immunological role for the later development of CAN comes from the observation that the length of transplant survival is inversely related to the number of human leukocyte antigen (HLA) mismatches carried by the graft, with a step-wise decrease in graft half-life as the number of HLA mismatches increases<sup>193-196</sup>. The best results are obtained with HLA-identical siblings<sup>197,198</sup> with some authors reporting the complete absence of CAN in the recipients of HLA-identical donors<sup>199</sup>. Mismatches at the HLA-DR locus are associated with a relatively poor prognosis compared to mismatches at the HLA-A and B loci and it is for these reasons that HLA matching has formed the basis of the kidney allocation process.

Nevertheless, that living donor allografts, irrespective of HLA mismatching, do better than cadaveric allografts emphasizes the importance of alloantigen-independent factors in the aetiology of CAN<sup>170,200</sup>. Indeed, the early work of Glasscock et al<sup>201</sup> demonstrated the failure of 40% of kidney transplants between identical twins at 10 years, with the kidneys exhibiting histopathological changes in the graft including arterial obliteration and glomerulosclerosis, consistent with the changes of CAN. This is supported by experiments in rodent models suggesting that isografts can develop changes consistent with CAN, albeit less rapidly than allografts<sup>202</sup>. Many alloantigen-independent factors may contribute to the differences observed in graft outcome. Indeed, cadaveric organs have been subjected to a multitude of injurious effects all of which may play a role in the pathogenesis of CAN<sup>190,203-205</sup>. Factors related to the donor such as donor hypertension and diabetes probably play a role in the outcome of the graft and it has further been shown that age, race and sex<sup>206</sup> of the donor and donor-recipient size mismatching can impact on the long term outcome of the transplanted kidney. Furthermore, that cadaveric donors have almost invariably sustained a huge central nervous system (CNS) insult prior to death renders their condition far from any physiological norm. There is evidence to suggest that the upregulation of cytokines, chemokines and adhesion molecules occurs following a brain injury with a rapid leukocyte infiltration and upregulation of their inflammatory products<sup>207-209</sup>. Furthermore, brain death includes a period of autonomic storm and changes associated with early chaotic swings in vascular

resistance and blood pressure with altered perfusion of peripheral tissue. Thus, there is the potential for the donor organs to be subjected to severe ischaemic injury prior to their removal. Indeed, possibly as a result of this severe ischaemic injury, the incidence of acute tubular necrosis (ATN) with delayed graft function is frequently increased in engrafted kidneys harvested from unstable brain-dead donors compared to those from living donors<sup>210,211</sup>. Other factors that may contribute to the long-term function of the cadaveric allograft include the inevitable effects of cold ischaemia i.e. following donor nephrectomy, and ischaemia-reperfusion injury<sup>212</sup>. In the living donor, the cold ischaemic time may be less than one hour; this is rarely the case for recipients of cadaveric kidneys. In experimental models, Tullius and others have demonstrated that prolonged ischaemia and delayed graft function result in functional and morphological changes consistent with CAN<sup>212-214</sup>. Specifically, some authors have postulated that cold ischaemic time may contribute to 40-45% of the vasculopathy present<sup>215</sup>. Others have suggested that cold ischaemia may play a greater role in the development of tubulointerstitial damage, with allogenicity proposed to play a greater role in the development of the vasculopathy seen in CAN<sup>216</sup>. Infection in general but particularly, cytomegalovirus (CMV) infection may also play a role in the development of chronic graft failure<sup>217</sup>.

Factors in the recipient may also impact prognostically on the allograft. These include the effects of hypertension<sup>218</sup> and lipid abnormalities, the latter, notably elevated cholesterol and triglycerides, believed to play a role in the vascular changes seen in CAN<sup>219,220</sup>. Whether this occurs through the upregulation of growth factor receptors on smooth muscle and other cells as has been postulated by some authors<sup>221</sup>, is not clear. A recent report has suggested that the oxidation of LDL lipids may stimulate fibrogenic cytokine formation and, in particular, TGF- $\beta$  from macrophages<sup>222</sup>.

### **1.3.3 Histopathological Changes in CAN**

CAN is characterised by a variety of histopathological changes which, once established, are invariably associated with graft loss. The dominant underlying pathological process appears to be fibrosis, with chronically failing allografts



demonstrating an excess of extracellular matrix (ECM) proteins. Indeed, the degree of fibrosis seems to provide the best morphological correlation with renal function and prognosis<sup>223,224</sup>. The hallmarks of chronic rejection first described by Hume in 1955<sup>225</sup> still form the basis of the Banff classification for renal transplant pathology used today. The Banff classification<sup>226,227</sup> grades “chronic transplant nephropathy” changes within the four structural compartments of the kidney, vascular, glomerular, interstitial and tubular lesions and it is the severity of these changes that impacts significantly on the prognosis of the graft.

The vascular lesion (also referred to as transplant vasculopathy) is common to all forms of chronic allograft pathology, irrespective of the organ involved and is illustrated in Figure 3.1e. It is characterised by perivascular inflammation, arteriosclerosis and concentric intimal fibrosis affecting large parts of the arteries and arterioles of the allograft. All arterial vessels may be affected including the small pre-glomerular arterioles, the interlobular arteries and, occasionally, the renal artery itself. The initial injury to the endothelium appears to be critical in the triggering of the cycle of events leading to CAN<sup>228</sup> and is likely to be initiated by both immune and alloantigen-independent factors as outlined above. Endothelial inflammation is associated with vacuolisation of the endothelial cells and their detachment from the basement membrane. Underlying adhesion molecules such as ICAM-1 and VCAM-1 are exposed<sup>229</sup> and this, together with the exposure of antigens, may attract inflammatory cells into the graft through the release of cytokines and growth factors<sup>230</sup>. The inflammatory infiltrate consists mainly of T cells and macrophages all of which may contribute to the luminal occlusion<sup>231-234</sup>. Frank endothelial inflammation, representing acute on chronic changes, may be seen in more than 50% of acute rejection biopsy specimens and represents a highly significant adverse prognostic feature compared to the features of a predominantly tubulointerstitial rejection<sup>192,235</sup>, rendering episodes of acute rejection less responsive to steroids. Endothelial inflammation encourages fibromuscular proliferation and the deposition of extracellular matrix contributing to the formation of a neointima and narrowing of the vessel lumen<sup>233,236</sup>. Eventually, obliteration of the allograft arteries occurs<sup>237</sup>. Indeed, it is likely that many of the glomerular and tubular changes seen in CAN

occur as a result of ischaemia secondary to the vascular injury, events correlating well with deteriorating renal function.

The glomerular changes occurring in CAN are less well defined than the changes seen in the vessels and are illustrated in Figure 3.1f. Glomerular tuft collapse, glomerular hypertrophy and atrophy may all occur<sup>238</sup> and are associated with expansion of the mesangium. There is often glomerular basement membrane duplication associated with scarring and adhesion<sup>239</sup>. Focal glomerulosclerosis is often seen<sup>240</sup>.

The tubular changes in CAN, including dilatation and atrophy, are often patchy with affected tubules being interspersed with normal or hypertrophied tubules. The interstitium, often oedematous early in the development of CAN, later becomes fibrous with a patchy inflammatory cell infiltrate composed predominantly of macrophages<sup>241</sup>, plasma cells, mast cells<sup>242</sup> and lymphocytes<sup>243,244</sup>. The inflammatory cell infiltrate is important in the development of CAN as evidenced by studies demonstrating that the onset and pace of CAN are slowed if the infiltration of mononuclear cells is inhibited<sup>245</sup>.

#### **1.3.4 Macrophages and macrophage products in CAN**

Macrophages and T cells<sup>246</sup> are the predominant cell types seen in the tubulointerstitial inflammatory cell infiltrate and, indeed, recipient monocytes and lymphocytes are the first cells to infiltrate the graft after transplantation. T lymphocytes are consistently present in low numbers and may provide continuous background immunological activity but the infiltration of monocytes into the graft relatively early following transplantation may be critical to graft outcome<sup>237,247,248</sup>. Acute rejection is accompanied by an increase in infiltrating monocytes and macrophages in renal allografts<sup>249-251</sup> and it has been suggested that macrophages formed the largest components of infiltrating cells in acute as well as chronic transplant pathology, with some authors suggesting that macrophage infiltration may prove a useful marker of acute rejection<sup>252</sup>, perhaps more useful than markers of

lymphocyte activity<sup>253</sup>. In the chronically failing allograft, macrophage infiltration and activity correlates directly with graft survival<sup>241,247,248</sup>. In one study, the number of infiltrating interstitial macrophages was markedly higher in the chronically failing allograft compared to a control group having stable renal graft function for more than 5 years<sup>351</sup>. In the same study, numbers of infiltrated glomeruli were also significantly higher<sup>254</sup>. By producing chemokines and cytokines such as IL-1, IL-6, TNF $\alpha$ , RANTES, MCP-1 and iNOS<sup>255</sup> and interacting with T cells, activated macrophages are important mediators in the development and amplification of the inflammatory response. Importantly, they are key mediators in fibrosis providing a large number of profibrotic factors and ECM proteins<sup>256,257</sup>. Indeed, the use of gamma lactone, a specific inhibitor of macrophage generation and effector activity *in vitro*<sup>258</sup>, results in histologically normal glomeruli and arteries and the prevention of proteinuria.

In addition to cytokines, macrophages produce a number of growth factors, many of which are implicated in the development of CAN<sup>259,260</sup>. These include platelet-derived growth factor (PDGF), a potent growth factor for smooth muscle cells<sup>244,261</sup>. That PDGF may play a significant role in the development of CAN has been highlighted recently by studies showing that the inhibition of PDGF prevents the development of CAN in a rat model<sup>262</sup>. Other macrophage-derived growth factor products upregulated in CAN include insulin-like growth factor-1 (IGF-1), a weak mitogen of smooth muscle cells which may enhance the mitogenic effects of PDGF, and fibroblast growth factor (FGF) which appears to have a crucial role in the regulation of media smooth muscle cell proliferation<sup>263</sup> and the glomerular lesions associated with CAN<sup>264</sup>. The macrophage product, VEGF, is a potent endothelial cell mitogen, angiogenic factor and enhancer of vascular permeability. It is also chemotactic for monocytes. It is upregulated in the interstitium in chronic renal allograft rejection and in the interstitium of kidneys with chronically reduced blood flow, where its expression co-localises with CD68-positive macrophages<sup>265,266</sup>. It is also upregulated in the glomeruli and vascular compartments of patients with chronic rejection although overall VEGF protein and mRNA expression is decreased in the chronically failing allograft as it is in other forms of chronic renal disease<sup>267</sup>.

TGF- $\beta$  is believed to be perhaps the single most important mediator of the fibrosis seen in CAN, with its expression being shown to be a significant correlate of interstitial fibrosis<sup>268-270</sup>, glomerulosclerosis<sup>271</sup>, vasculopathy<sup>177</sup> and declining renal function<sup>272</sup>. TGF- $\beta$  is a multifunctional growth factor produced by a number of different cell types in response to a range of different stimuli<sup>107</sup>. TGF- $\beta$  has 3 isoforms whose biological properties are almost identical; TGF- $\beta$ 1, 2 and 3. TGF- $\beta$ 1 is a potent chemotactic agent for fibroblasts and, through the enhanced production of acidic fibroblast growth factor stimulates fibroblast proliferation<sup>273</sup>, an effect contributed to by other activated macrophage and lymphocyte products such as TNF $\alpha$  and IL-1 $\beta$ . TGF- $\beta$  enhances the synthesis of a number of extracellular matrix proteins including collagen, fibronectin and proteoglycans<sup>274</sup> and it inhibits the degradation of newly formed matrix proteins<sup>275,276</sup>. TGF- $\beta$ 1 is normally transiently upregulated in response to tissue injury and is essential in driving normal wound healing<sup>277,278</sup>. However, fibrosis represents a pathological excess of the normal process of tissue repair and sustained or increased synthesis of TGF- $\beta$  results in a shift towards accumulation of ECM and inhibition of its degradation. The administration of intravenous TGF- $\beta$  induces serious systemic effects in rats including marked fibrosis in the kidneys and liver and at the injection site<sup>271</sup>. Furthermore, in mice given TGF- $\beta$ 1 intraperitoneally for 10 days, severe cachexia and generalised tissue fibrosis develop<sup>279</sup>. TGF- $\beta$ 1 plays other roles of relevance to the development of CAN. For example, it regulates PDGF (in smooth muscle cells and fibroblasts), FGF (in endothelial cells) and TNF $\alpha$  and IL-1 (in monocytes)<sup>280,281</sup>. It also induces the transdifferentiation of proximal convoluted tubule epithelial cells in the kidney into myofibroblasts<sup>282</sup>, cells having properties of both smooth muscle cells and fibroblasts. In addition, it inhibits lymphocyte proliferation and effector function<sup>283</sup> and, furthermore, is capable of exhibiting positive autoregulation of its own synthesis, thereby augmenting its fibrogenic actions<sup>284</sup>. The commonly employed immunosuppressive agents ciclosporin and tacrolimus also upregulate TGF- $\beta$ <sup>285,286</sup>.

## **1.4 Macrophage Activation**

### **1.4.1 Introduction**

As discussed above, macrophages are an integral component in the inflammatory cell infiltrate seen in CAN. Furthermore, they provide a critical link between the innate and adaptive immune systems acting as a stimulus for T cell activation and effector function. Macrophages have been shown to express the receptor for Shh, Ptc, and, since both epithelium and CD4+ T cells express Shh, there is clearly the potential for a number of interactions to occur at sites of epithelial damage and immune cell infiltration through the Shh signalling pathway. Given that macrophages may be able to respond to a Shh signal, an area that has been explored in Chapter 5 of this thesis, this part of the introduction introduces the concept of macrophage activation. The aim of this section of the introduction, like the following section on T cell activation, however is not to comprehensively review these areas and the reader is directed to a number of reviews covering the area<sup>287-290</sup>. Rather, these sections will concentrate on the areas of macrophage and T cell activation relevant to the experimental procedures described later in this thesis.

### **1.4.2 The Innate Immune System**

The innate immune system represents the first line of defence to attack by pathogens. Pathogens that breach the epithelial barrier are intercepted by macrophages and dendritic cells capable of binding to and phagocytosing a wide variety of pathogens. Subsequent processing of the antigen allows presentation to lymphocytes, thus instigating adaptive immunity in the form of specific T and B cell effector mechanisms. The initial recognition of pathogens is mediated by a number of different pattern recognition receptors (PRRs) on the host phagocyte and these are responsible for initiating acute inflammatory responses against invading pathogens<sup>291</sup>. These receptors recognise pathogens by detecting highly conserved repeating patterns (pathogen associated molecular patterns, PAMPs)<sup>292</sup> which are essential for survival of the pathogen. The PRRs represent a diverse group of

receptors which includes scavenger receptors<sup>293,294</sup>, the type 3 complement receptor, CD36<sup>295</sup>, DC-SIGN<sup>296</sup> and the mannose receptors<sup>297</sup>. In addition to these receptors, there is a further large group of PRRs, the Toll-like receptors (TLRs), which have transformed our knowledge of innate immunity<sup>298-300</sup> and will be discussed in greater detail below. PRRs instigate the uptake and processing of the pathogen by the phagocyte, allowing pathogenic antigens to be presented on the surface of the host antigen presenting cells (APCs) in conjunction with MHC molecules thus allowing activation of specific T and B lymphocytes to occur and the triggering of the adaptive immune response.

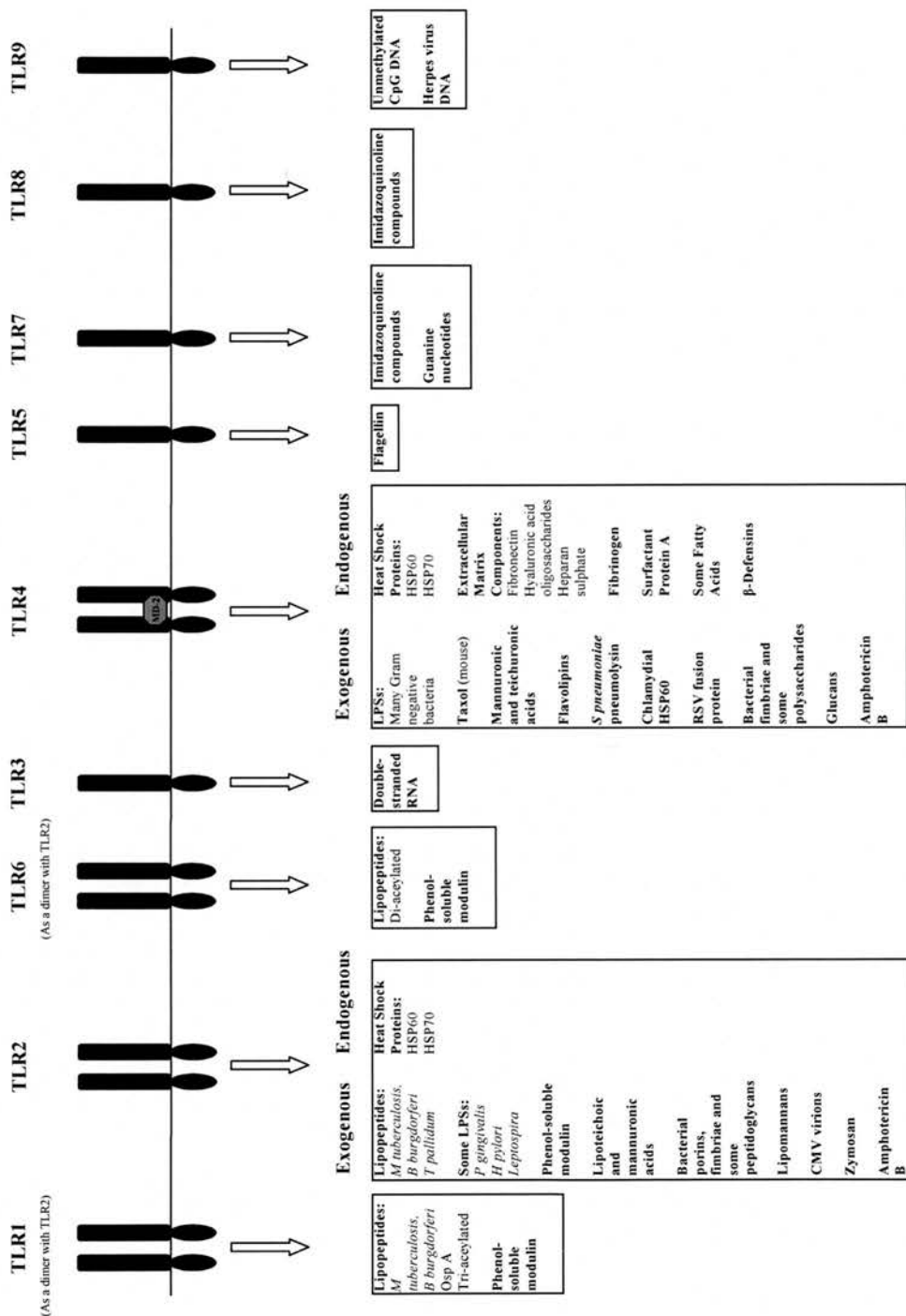
### 1.4.3 The Toll-Like Receptors

Since the discovery of the human homologue of *Drosophila* Toll<sup>301</sup>, there has been a rapid expansion in the number of TLRs identified<sup>291,299</sup>. To date, 10 TLRs have been reported and these are differentially expressed in immune cells, including macrophages, dendritic cells and T cells, and a number of non-immune cells including endothelial cells and epithelial cells. Nearly all of the TLR agonists so far identified are bacterial in origin reflecting the variety of bacterial components that can be recognised by the innate immune system.

Of the TLRs, most attention has focused on TLR4, the main transducer of the lipopolysaccharide (LPS) signal. LPS is an extremely potent stimulant of immune responses and is the principal cause of septic shock in humans<sup>302</sup>. The potency of LPS is highlighted by studies showing that as little as 10pg/ml LPS can induce significant cytokine production<sup>303,304</sup>. Although TLR4 is the best characterised of the TLRs and the main receptor responsible for the signal transduction of LPS, other TLRs are recognised to play important roles in the recognition of bacterial components<sup>299,305</sup>. For example, TLR2 recognises lipoproteins<sup>306</sup>, glycolipids, peptidoglycans<sup>307</sup>, lipoteichoic acid<sup>308</sup> and a variety of other Gram positive bacterial products. TLR3 recognises double-stranded RNA<sup>309</sup> and TLR5 and TLR9 recognise flagellin<sup>310</sup> and bacterial DNA respectively. Indeed, bacterial agonists have now been identified for nearly all of the TLRs (Reviewed in <sup>299,300,305</sup>)(See Figure 1.2)



emphasizing the important role of these molecules in the recognition of pathogens by cells of the innate immune system. Interestingly, there have been a number of reports in the literature proposing that, aside from exogenous agonists for the TLRs, a number of endogenous agonists exist. Proposed endogenous agonists for the TLRs are shown in Figure 1.2. The interest in these endogenous agonists for TLR4 has been dramatic and unlike that seen for any other TLR. These structurally and functionally unrelated endogenous agonists including the extracellular matrix components fibronectin<sup>311</sup>, hyaluronic acid<sup>312</sup>, heparan sulphate<sup>313</sup>, fibrinogen<sup>314</sup>, surfactant protein A<sup>315</sup>, fatty acids,  $\beta$ -defensins<sup>316</sup> and members of the heat shock protein (HSP) family; HSP60<sup>317-321</sup> and HSP70<sup>322-324</sup>. These endogenous agonists are purported to activate macrophages and dendritic cells in a similar way to the bacterial TLR4 agonists, inducing the expression of proinflammatory cytokines and costimulatory molecules discussed further below. Why endogenous agonists and, indeed, so many structurally and functionally different agonists should induce similar patterns of effector function in macrophages and dendritic cells is not clear. In the case of the heat shock proteins, whose primary function is to chaperone proteins (Reviewed in <sup>325</sup>), it is proposed that the proteins are released from damaged or dying cells<sup>326</sup> thus providing a “danger signal” to the immune system<sup>327</sup>. Other investigators have been more sceptical regarding the proposed endogenous TLR4 activators positing that some, if not all, of the effects seen in response to the proposed TLR4 agonists may be attributable to bacterial components of the proteins used to explore their effects *in vitro*. Indeed, some authors have reported on studies demonstrating that endotoxin-free HSPs fail to induce a significant macrophage effector response<sup>303,328,329</sup>.



**FIGURE 1.2**

## The TLRs and their agonists

Numerous agonists have now been identified for the TLRs. In the most part, the agonists are bacterial in origin reflecting the critical importance of the TLRs in the innate response to bacterial pathogens.



#### 1.4.4 The Macrophage Effector Response

The TLRs show homology with the IL-1 receptor. Activation of the TLRs and the IL-1 receptor results in the polymerisation of the TLRs or IL-1 receptor and the interaction with an adaptor protein. Of the adaptor proteins, MyD88 was the first to be associated with TLR signalling<sup>330</sup> and is the best characterised. It appears to play a critical role in the response to TLR agonists and IL-1<sup>331</sup>. It is generally believed that, following activation, MyD88 recruits a serine-threonine kinase – the IL-1 receptor associated kinase (IRAK). This is activated by dephosphorylation and associates with TRAF6 with the subsequent activation of the JNK and NF- $\kappa$ B pathways<sup>332-334</sup>. It has been suggested that all TLR proteins may use MyD88<sup>330,332</sup> and, indeed, macrophages from mice deficient in MyD88 fail to generate a significant inflammatory response to a variety of TLR agonists

However, that TLR4 may also use other adaptor proteins was soon realised following the observation that a TLR4 agonist induced downstream pathway activation in MyD88-deficient mice<sup>331</sup>. Indeed, in these mice, LPS was also shown to induce activation of the downstream targets NF- $\kappa$ B and JNK albeit with delayed kinetics. Furthermore, this MyD88-independent activation pathway may have very distinct biological roles (Reviewed in <sup>305</sup>). For example, the expression of IFN- $\gamma$ -inducible genes, including IP-10 and IRG-1 by macrophages in response to LPS appears to be TLR4-dependent but MyD88-independent<sup>305</sup>. It has also been posited that the LPS-induced maturation of dendritic cells (DCs) may, at least in part, depend on MyD88-independent pathways as evidenced by studies in which enhanced expression of costimulatory molecules and increased T cell allo-stimulation was observed in DCs from MyD88-deficient, but not TLR4 deficient mice<sup>335,336</sup>. One of the adaptor proteins implicated in MyD88-independent signalling is the TIR domain-containing adapter protein (TIRAP)<sup>337</sup>, a protein also known as MyD88 adapter-like (Mal)<sup>338</sup>. In contrast to MyD88, TIRAP does not appear to participate in IL-1 signaling<sup>337</sup>. Other adaptor proteins may also play a role in TLR signalling. For example, Toll-interacting protein (TOLLIP), a protein first identified as a component of the IL-1

receptor pathway<sup>339</sup>, may be important as a negative regulator of the TLR signalling pathways<sup>320,340</sup>.

As a result of TLR activation, a number of downstream pathways are activated. The end result of activation of these pathways is the production of an effective macrophage capable of both dealing effectively with elements recognised by the body to be pathogenic or non-self and, also, activating T cells thus generating an efficient adaptive immune response. The activation of T cells is discussed in greater detail in Section 1.4.

Of the downstream pathways activated as a result of TLR activation, the highly conserved and structurally related NF- $\kappa$ B proteins play a central role in several immune and inflammatory processes<sup>341</sup>. In resting cells, NF- $\kappa$ B proteins are sequestered in the cytoplasm by their inhibitors I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$ . Activation of the macrophage by LPS or other stimuli activates the I $\kappa$ B kinase complex (IKK) which phosphorylates I $\kappa$ B. Phosphorylated I $\kappa$ B becomes degraded thus releasing NF- $\kappa$ B and allowing the translocation of NF- $\kappa$ B/Rel complexes such as p50/p65 to the nucleus where they can activate the transcription of a number of genes playing roles in the innate immune response. In addition to the activation of NF- $\kappa$ B, TLR signalling also initiates signalling through phosphoinositide-3-kinase (PI3K) and the mitogen-activated protein kinases (MAPK) signalling cascades including the ERK, JNK and p38 pathways with the subsequent direct or indirect activation of a series of transcription factors including Elk-1, c-Jun, c-Fos, ATF-1 and 2, SRF and CREB<sup>342</sup>. LPS induces a large number of genes that express inflammatory mediators and enhance antigen presenting activity<sup>291,300,305</sup>. These include cytokines (e.g. IL-1, IL-2, IL-6, IL-12, TNF $\alpha$  and GM-CSF), chemokines (e.g. IL-8, monocyte chemotactic protein (MCP-1), Regulated on activation normal T cell expressed and secreted (RANTES), Macrophage inflammatory protein (MIP)-1 $\alpha$  and -1 $\beta$ , melanoma growth stimulatory activity (GRO), IFN- $\gamma$ -inducible protein-10 (IP-10)), adhesion molecules, growth factors (e.g. VEGF) co-stimulatory molecules and inducible enzymes (e.g. iNOS)<sup>341,342</sup>.

It was once thought that LPS and other TLR agonists induced the same effector response in responding cells. However, it is now increasingly being recognised that activation via different TLR pathways can induce differential effector functions<sup>343-347</sup>. The MyD88-dependent pathway, together with an apparently increasing group of MyD88-independent pathways adds significant diversity to the TLR effector response. Adding to this diversity are a number of other interesting reports in the literature. For example, there are reports suggesting that synergy may occur between TLRs<sup>348,349</sup> and, furthermore, that TLR4 signalling may upregulate the expression of TLR2<sup>350</sup>. Together, these factors add a further dimension to the investigation of TLR mediated signalling suggesting that, far from serving a non-specific effector function as previously thought, the innate immune system is potentially capable of demonstrating both specificity and selectivity in its response.

A further element of specificity in the macrophage response is illustrated by the recognition that different combinations of stimuli may also give rise to macrophages with different effector functions. The phenotypes include the classically activated macrophage, the alternatively activated macrophage, the type II activated macrophage and other phenotypes generated following treatment with IL-10, steroids or the uptake of apoptotic cells. These phenotypes have been reviewed elsewhere<sup>287,351,352</sup> and will only be discussed briefly here. Classical macrophage activation involves 2 key signals, the first provided by TLR activation and the second by IFN- $\gamma$  provided, *in vivo*, by T cells or natural killer cells. Proinflammatory cytokines (including IL-1 $\beta$ , IL-6, IL-8, MCP-1, TNF $\alpha$ ), nitric oxide and reactive oxygen species are induced and costimulatory and MHC class II molecules are upregulated. Classical activation plays a significant role in initiating the adaptive immune response<sup>287</sup>. In contrast, alternative macrophage activation occurs typically following exposure to IL-4 or IL-13<sup>353,354</sup>. Nitric oxide, TNF $\alpha$  and IL-6 production are decreased on a background of increased mannose receptor and MHC class II expression. These macrophages are proposed to play a role in tissue repair processes<sup>355</sup>. The recently identified “type II-activated macrophage”<sup>352</sup> expresses upregulated MHC class II, IL-10, IL-6 and TNF $\alpha$  but reduced IL-12 expression. The phenotype was found to be induced by stimuli such as LPS or CD40 ligand together

with IgG immune complexes<sup>356</sup>. Other macrophage phenotypes may be induced by stimuli such as apoptotic cell uptake, IL-10 or steroid treatment<sup>351</sup>.

## **1.5 T Cell Activation**

### **1.5.1 Introduction**

As highlighted in the previous section, macrophages are central to an efficiently functioning innate immune response. In addition, as antigen presenting cells, they present a critical link between the innate and adaptive immune responses.

Furthermore, the recent reports suggesting that macrophages express the receptor for Shh, Ptc, and that Shh signalling is important in the generation of a CD4<sup>+</sup> T cell effector response highlight the potential role for Shh signalling in the link between the innate and the adaptive immune responses. Thus, in the section of the introduction that follows, areas of T cell activation relevant to the context of this thesis will be explored further.

Following development in the thymus, mature T cells circulate between the blood and lymphoid tissue as naïve T cells. On encountering a specific antigen in association with an antigen presenting cell (APC), a naïve T cell is activated. This encounter halts the circulation of the T cell, inducing its proliferation and the induction of an effector function that allows the T cell to deal appropriately with the encountered antigen. The effector cells that result from this process re-enter the blood and migrate to the sites of antigen invasion. Conversely, naïve T cells which do not recognise the specific antigen continue to recirculate between the blood and lymphoid organs<sup>357,358</sup>. In addition to this primary immune response, immunological memory is generated in the form of long-lived memory T cells which allow an accelerated response to subsequent encounter with the same antigen.

### **1.5.2 Antigen presentation**

For T cell activation to occur, the T cell recognises foreign antigen in association with a major histocompatibility (MHC) complex present on APCs. Several cell types can function as APCs including dendritic cells (DCs), macrophages and B cells. Mature DCs are the most potent activators of naïve T cells, playing a role in the

presentation of bacterial, viral and fungal antigens to T cells. Both immature dendritic cells and resting macrophages originate from myeloid precursors which lack high levels of MHC and circulate in the blood to reach peripheral tissues. In the periphery, these cells are capable of phagocytosing antigenic material, an action effected through a range of receptors on the cell surface most of which recognise microbial products. Following phagocytosis, the antigenic material is processed. Signalling triggered by the process of phagocytosis induces the upregulation of MHC molecules and it is this complex of MHC and antigen that is recognised by the T cell receptor (TCR) complex and will be discussed in more detail below.

In addition to a role in the T cell response to pathogenic antigens, T cell activation also forms a vital component of the host response to alloantigen in the context of transplantation. In this respect, donor organ alloantigens can be presented to recipient naïve T cells in 2 ways. In the first of these, termed direct allorecognition, donor APCs (also termed passenger leukocytes) migrate from the donor organ and present donor alloantigens to naïve host T cells in the draining lymph nodes<sup>359</sup>. Passenger leukocytes with the properties of immature dendritic cells<sup>360</sup> migrate very rapidly out of an organ following transplantation and quickly mature into APCs capable of stimulating naïve T cells<sup>361</sup>. Activated cytotoxic T cells return to the graft which can then be attacked directly.

A further mechanism, termed indirect recognition, also plays a role in alloantigen recognition. In this mode of alloantigen recognition, recipient APCs take up donor alloantigens and present them to naïve recipient T cells, a mechanism more in keeping with the orthodox modes of antigen presentation observed during the presentation of pathogenic antigens. Since the MHC class II pathway deals primarily with exogenous proteins, it seems likely that most allo-MHC peptides might be presented in the context of MHC class II. However, some crossover between Class II and I pathways may also exist<sup>362</sup>. The relevance of the indirect pathway to acute rejection has not been completely established although it has been posited that this pathway may play more of a role in chronic rejection processes where the ongoing

presence of antigenic stimulus could continue to drive a background immune response<sup>363</sup>.

### **1.5.3 T cell receptor mediated activation**

For T cell activation to occur, only very small numbers of antigen-MHC complexes are required. Indeed, as few as 1-400 complexes may be necessary to initiate recognition by the TCR<sup>364-367</sup>. The TCR binding site comprises heterodimers of variable TCR  $\alpha\beta$  and  $\gamma\delta$  receptor chains<sup>368</sup>. The TCR associates with the CD3 complex, a structure consisting of invariant  $\gamma$ ,  $\delta$  and  $\epsilon$  chains and a homodimeric  $\zeta$  chain. The TCR confers specificity of MHC-antigen binding. Conversely, the  $\zeta$  chains of the CD3 complex transduce activation signals to the T cell. Within the chains of the CD3 complex exist amino acid sequences termed Immunoreceptor Tyrosine-based Activation Motifs (ITAMs). Each CD3 chain contains one ITAM and each  $\zeta$  chain contains three. Tyrosine phosphorylation of these motifs initiates TCR-associated signal transduction and the downstream activation of intracellular pathways<sup>369</sup>. CD8 and CD4 are accessory molecules to the TCR-mediated activation process. They bind to MHC molecules at a different site to the TCR and form an important part of the TCR complex, increasing the affinity of interaction between the T cell and the APC. CD8 and CD4 also define the MHC restriction of the T cell (recognising class I and class II MHC respectively) and have a role to play in the optimisation of T cell effector function. Although some similarities exist between CD4 and CD8 T cell activation events, the following discussion regarding T cell activation and T cell effector function will be directed at events occurring in the CD4+ T cell since these bear direct relevance to the work presented in this thesis.

In the CD4+ T cell, TCR activation is not sufficient to generate optimal T cell activation. A second co-stimulatory signal to the T cell is also required. Indeed TCR activation in the absence of a second costimulatory signal results in T cell unresponsiveness or anergy<sup>370</sup>. Several molecules are likely to contribute to costimulatory signals and include the B7 family of molecules, B7-1 (CD80) and B7-2 (CD86). CD80 is absent and CD86 is expressed only at low levels on resting



APCs<sup>371</sup> although both are upregulated alongside MHC molecules when APCs take up and process antigen in the periphery.

The receptor for the B7 molecules on the T cell is CD28<sup>372,373</sup>, a glycoprotein expressed as a homodimer and present on 95% CD4+ T cells and 50% CD8+ T cells<sup>374-376</sup>. CD28 interacts with B7 ligands through a MYPPPY recognition motif in its extracellular domain<sup>373</sup>. In contrast to the TCR signalling through ITAMs, CD28 signals are mediated through phosphorylated tyrosines and proline-rich motifs in its 41-residue cytoplasmic tail<sup>377</sup>. Costimulation through CD28 ligation synergises with TCR engagement, lowering the threshold for activation to occur and resulting in a rapid increase in the tyrosine phosphorylation of CD28<sup>378</sup>. The synergistic signal provided by CD28 appears to be greatest in instances of low TCR occupancy. In contrast, very strong TCR signals and T cells exposed to antigen appear to be less CD28-dependent<sup>379</sup>.

Aside from CD28, other costimulatory molecules are likely to also contribute to T cell signalling. Such molecules include Inducible co-stimulator (ICOS), a T cell costimulatory protein related to CD28<sup>380</sup> that is expressed on resting but not activated T cells<sup>380</sup>, and members of the TNF receptor family including CD40 Ligand (CD40L/CD154)<sup>381</sup>, OX-40 (CD134)<sup>382</sup>, a potent inducible costimulatory molecule<sup>383</sup>, 4-1BB (CD137) and CD27<sup>384</sup>, a molecule implicated in T cell activation, T cell development and T cell dependent antibody production by B cells. Other co-stimulatory molecules on the T cell surface capable of upregulating TCR signals include CD5, CD9<sup>385</sup> and CD2<sup>386</sup>.

The initial interaction of the TCR with the MHC-antigen complex triggers a tyrosine phosphorylation cascade that triggers multiple branching signalling pathways<sup>387-389</sup>. These early signals may be sufficient to trigger some effector functions such as killer T cell activity but other functions such as T cell proliferation and cytokine effector function appear to require a more prolonged TCR engagement in keeping with minutes or hours of T cell-APC contact. In this regard, the formation of the immunological synapse appears to be central to the T cell TCR-MHC interaction<sup>390</sup>.



The mature T cell synapse is thought to comprise a central cluster of TCRs surrounded by a ring of integrins, mainly LFA-1<sup>390,391</sup>. Although the TCR plays a key role in the initiation and maintenance of an activated state, it may be that the integrins and other T cell surface molecules such as the costimulatory molecules play important roles in recognition. One commonly held view is that the T cell first probes the APC through pseudopodial extensions and, following recognition, integrins allow the T cell to flatten against the APC with the formation of the immunological synapse. Although synapse formation does not appear to be necessary to trigger signalling through the TCR, it may be needed to sustain the activated T cell response. The combination of TCR/CD3 signalling, co-stimulation and integrin interactions appear to initiate the active transport of TCR molecules from all over the T cell to the immunological synapse allowing concentration of the signalling components and optimal stimulation of the T cell by the antigen-MHC complex<sup>392,393</sup>. Studies investigating the mechanisms underlying synapse formation have suggested that lipid rafts may serve as a platform for T cell signalling<sup>394-396</sup>. Indeed, it would seem that, following TCR engagement, the TCR  $\zeta$  chain and downstream transducers are tyrosine phosphorylated and stabilised within lipid rafts allowing further rafts to be recruited with the formation of larger rafts at the TCR contact point (cap). This raft coalescence appears to be critical for the generation of a sustained TCR signal, effective signal transduction (reviewed in<sup>397,398</sup>) and the subsequent production of IL-2<sup>399</sup>.

TCR ligation, in association with a second costimulatory signal, initiates a complex series of intracellular activation events which culminate in the activation of at least three central signalling pathways; nuclear factor of activated T cells (NFAT)<sup>400,401</sup>, activator protein 1 (AP-1)<sup>402</sup> and nuclear factor  $\kappa$ B (NF $\kappa$ B)<sup>403</sup>. The activation of phospholipase C- $\gamma$  (PLC- $\gamma$ ) is central to the activation of the NF $\kappa$ B and NFAT pathway. PLC- $\gamma$  activity results in the generation of inositol-3,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>). IP<sub>3</sub> promotes calcium release from the endoplasmic reticulum. The elevation in intracellular calcium activates calmodulin, a calcium binding protein and calcineurin, a serine/threonine protein phosphatase<sup>404</sup>. Calcineurin is activated by the binding of

calcium and calmodulin, and transmits signals to the nucleus through the dephosphorylation and translocation of NFAT transcription factors. NFAT is central to lymphocyte development and proliferation as evidenced by studies in which targeted disruption of NFAT leads to defective development and proliferation<sup>405,406</sup>. Ciclosporin and tacrolimus (FK506) inhibit the phosphatase activity of calcineurin resulting in the relocalisation of NFAT to the cytosol, loss of its DNA-binding activity and consequent inhibition of T cell proliferation, differentiation and effector function. It is for this reason that calcineurin inhibitors are often used in the immunosuppressive regimen for recipients of solid organ transplants.

#### **1.5.4 The CD4+ T cell effector response**

Activation of the downstream pathways outlined above serves to control a number of T cell events and effector processes. Specifically, T cell activation induces the clonal expansion of T cells and promotes T cell differentiation into effector cells<sup>407,408</sup>. The clonal expansion of T cells occurs primarily through an increase in IL-2 secretion<sup>409,410</sup>. Enhanced IL-2 production is accompanied by associated increases in IL-2 receptor (IL-2R) expression<sup>411</sup>. The IL-2R comprises 3 chains:  $\alpha$ ,  $\beta$  and  $\gamma$  and the upregulated IL-2 production is associated with a concomitant increase in the expression of the  $\alpha$  chain of the IL-2 receptor. This, when associated with the  $\beta$  and  $\gamma$  chains, results in a receptor with high affinity for IL-2 allowing cells to rapidly respond to low levels of IL-2 and clonally expand and differentiate into effector T cells.

Following T cell activation, a range of different cytokines may be expressed and the profile demonstrated determines the effector function of a given CD4+ T cell. In the 1980s, it was proposed that, based on the cytokine expression profile, 2 broad populations of effector T cell were generated<sup>412,413</sup>. These were termed Th1 and Th2 CD4+ T cells. Although this classification is simplistic and increasingly believed to be inappropriate, indeed the given cytokine effector profile is likely to be a mixture of cytokines reflecting the heterogeneity of immune responses induced, it is still used to broadly describe the types of cytokine profile generated by different subsets of Th

cells. Both Th1 and Th2 cells appear to derive from a common Th0 precursor. Whether a Th1 or Th2 cell develops depends on the cytokine milieu in which the Th cell finds itself. The production of IL-12 by macrophages and dendritic cells will direct the Th0 cell towards a Th1 phenotype. Such cells produce relatively large quantities of cytokines important for cell-mediated immunity including IL-2, IFN- $\gamma$  and TNF. As outlined above, IL-2 is rapidly induced by T cell activation. It is primarily responsible for regulating T cell growth and clonal expansion<sup>414</sup>. It also stimulates B cell and natural killer (NK) cell growth. IFN- $\gamma$  has a number of effector functions. It activates macrophages, upregulates MHC class I and II expression and enhances their phagocytic and microbicidal activity. Furthermore, it activates neutrophils and NK cells and is also responsible for induction of antibody class switching to the IgG2a isotype in B cells<sup>415,416</sup>. IFN- $\gamma$  is induced not only by TCR-mediated activation<sup>414</sup> but also by other cytokines including IL-2, IL-12, IL-15, TNF. Together, the Th1 cytokines promote the development of cytotoxic T cells and facilitate antibody-dependent cellular cytotoxicity serving as effector mechanisms against intracellular pathogens. In contrast to the inflammatory cytokines induced in Th1 T cells, Th2 T cells secrete effector molecules concerned with regulation of Th1-mediated effects and the humoral immune response<sup>417</sup>. Th0 cells are polarised towards a Th2 phenotype when cultured in the presence of IL-4, the source of which may be a number of different cell types including naïve T cells and a small population of NK T cells. Cytokines produced by Th2 cells include IL-3, IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, cytokines important in mediating the B cell response. These cytokines stimulate B cell proliferation and synthesis of antibodies, promote the differentiation of B lymphocytes into antibody-secreting plasma cells and allow antibody class switching. These cytokines also allow B lymphocytes to activate eosinophils. Furthermore, IL-5 plays a role in enhancing IL-2-dependent T cell proliferation and differentiation although, since T cells do not express a clear receptor for IL-5, the mechanism by which this occurs is not clear. IL-10 is an important cytokine originally incorporated into the Th2 subset but produced by a number of cell types including macrophages, Th1 and Th2 CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells, NK cells, mast cells and B cells<sup>418,419</sup>. IL-10 is important for a number of reasons, not least because it functions as a significant down-regulator of

inflammatory responses. IL-6 is important in inducing B cells to differentiate into plasma cells and acts with cytokines such as IL-1 and IL-2 to induce T cell activation.

## 1.6 Hypotheses

The Shh signalling pathway has a number of roles in a variety of developmental processes. More recently, a growing body of evidence has evolved to suggest that activation of the Shh signalling may play a role in the adult. Recent reports in the literature have suggested that Shh signalling may play a role in inflammation and fibrosis and, further, that it may be important in the maintenance of peripheral immunity. Indeed, immune cells have been shown to express Shh signalling pathway components and further work from our laboratory has shown that the proliferation and cytokine effector function of CD4<sup>+</sup> T cells is modulated by Shh signalling. The work to date suggesting a role for Shh signalling in inflammation and fibrosis has concentrated on human lung disease and a murine model of lung fibrosis. Other conditions characterised by inflammation and fibrosis have not been investigated.

Chronic renal allograft nephropathy (CAN), as discussed earlier in this chapter, is characterised by interstitial fibrosis, tubular atrophy, vasculopathy and a mononuclear cell infiltration. The inflammatory cell infiltrate consists of mainly macrophages and T cells. TGF- $\beta$  and VEGF are among a number of factors produced by activated macrophages and both growth factors have been demonstrated to be upregulated in CAN. Both factors may also be downstream targets of Shh signalling. Therefore, kidney and the kidney affected by CAN prove interesting areas for further study. **Thus, the first hypothesis central to this thesis was that Shh signalling pathway components are expressed in and relevant to normal and CAN kidney.**

The second hypothesis evolves from the findings that macrophages and T cells express Shh signalling pathway components and that the addition of recombinant Shh to cultures of activated CD4<sup>+</sup> T cells modulated the proliferation and cytokine effector function of these cells. **Thus, the second hypothesis was that Shh signalling may be important in peripheral immunity.** Specifically, in this regard, the work presented here has questioned the possible role for Shh signalling in macrophages, an area which has, to date, not been explored. Furthermore, and drawing on the recently published reports suggesting a role for Shh signalling in

adult human and murine T cell effector function, this work has sought to further evaluate this area of investigation.

## **Chapter 2**

### **Materials and Methods**

## 2.1 Materials

All reagents were purchased from Sigma (Poole, Dorset, UK) unless stated otherwise.

### 2.1.1 Media

The following media were used for the tissue culture experiments:

#### **Serum-free IMDM culture medium for monocyte adherence**

500 ml IMDM (Gibco, Paisley, UK) with 50IU/ml penicillin/50µg/ml streptomycin (Gibco, Paisley, UK) and 2mM L-glutamine (Gibco, Paisley, UK). Stored at 4°C.

#### **Complete IMDM culture medium with 5% fetal calf serum (IMDM-FCS) for macrophage culture**

500 ml IMDM (Gibco, Paisley, UK) with 50IU/ml penicillin/50µg/ml streptomycin (Gibco, Paisley, UK), 2mM L-glutamine (Gibco, Paisley, UK) and 5% fetal calf serum (FCS) (F-9665). Stored at 4°C.

#### **Serum-free RPMI culture medium for monocyte adherence**

500 ml RPMI 1640 (R-0883) with 50IU/ml penicillin/50µg/ml streptomycin (Gibco, Paisley, UK) and 2mM L-glutamine (Gibco, Paisley, UK). Stored at 4°C.

#### **Complete RPMI culture medium with 5% human AB serum for T cell culture**

500 ml RPMI 1640 (R-0883) with 50IU/ml penicillin/50µg/ml streptomycin (Gibco, Paisley, UK), 2mM L-glutamine (Gibco, Paisley, UK) and 5% human AB (heat inactivated) serum (H-1513). Stored at 4°C.



**Complete DMEM for ACHN cell culture**

500ml DMEM with 100IU/ml penicillin/100µg/ml streptomycin (Gibco, Paisley, UK), 2mM L-glutamine (Gibco, Paisley, UK), 10% FCS and 1% Non-Essential Amino Acids (Gibco, Paisley, UK). Stored at 4°C.

**Complete DMEM for murine renal tubular epithelial cell culture**

500ml DMEM/F12 (Gibco, Paisley, UK) with 100IU/ml penicillin/100µg/ml streptomycin (Gibco, Paisley, UK), 2mM L-glutamine (Gibco, Paisley, UK), 1% ITS (insulin/transferrin/sodium selenite) supplement (I-3146), 30ng/ml dexamethasone (D-8893), 25ng/ml Epidermal Growth Factor (E-4127). Stored at 4°C.

**Complete HBSS**

500ml HBSS with 50IU/ml penicillin/50µg/ml streptomycin (Gibco, Paisley, UK). Stored at 4°C.

**2.1.2 Cell Separation and Stimulation Reagents**

The following reagents were employed in the isolation of specific cell populations from Buffy coats and for the cell stimulation experiments:

**MACS Buffer**

0.5% BSA (A-2153) in PBS (D-8537). Stored at 4°C.

**Red Blood Cell Lysis Buffer**

1mM  $\text{NH}_4\text{HCO}_3$  and 114mM  $\text{NH}_4\text{Cl}$ . Stored at 4°C.

**Collagenase Digestion Mixture**

20ml complete HBSS with 0.05% collagenase (C-5138) and 20µl (0.1%) DNase I (Roche 1284932). Sterile filtered prior to use. Stored at 4°C.

## **Freezing Mix**

10% DMSO in FCS

### **2.1.3 Flow Cytometry Reagents**

The following reagents were utilised for experiments involving flow cytometry:

#### **FACS Wash Buffer**

0.1% sodium azide, 0.2% bovine serum albumin (BSA) (A-2153) in PBS (D-8537).  
Stored at 4°C.

#### **FACS Fixing Reagent**

2% paraformaldehyde (P-6148) in PBS (D-8537). Stored at 4°C.

### **2.1.4 Immunoassay Reagents**

The following reagents were employed in Enzyme Linked Immunosorbent Assay (ELISA) experiments:

#### **ELISA Wash Buffer**

PBS tablets (Oxoid BR14) diluted in distilled water (1 tablet/100ml water) with 0.05% Tween-20 (P-7949). Stored at room temperature.

#### **ELISA Block Buffer (VEGF and Shh ELISA)**

1% BSA (A2153), 5% sucrose, 0.05% sodium azide in PBS. Stored at 4°C.

#### **ELISA Block Buffer (TGF- $\beta$ ELISA)**

5% Tween-20 (P-7949), 5% sucrose, 0.05% sodium azide in PBS. Stored at 4°C.

#### **ELISA Reagent Diluent (VEGF and Shh ELISA)**

1% BSA in PBS, pH 7.2-7.4, 0.2 $\mu$ m sterile filtered. Stored at 4°C.

**ELISA Reagent Diluent (TGF- $\beta$  ELISA)**

1.4% delipidised bovine serum (R&D Systems, Abingdon, UK), 0.05% Tween-20 (P-7949) in PBS, pH 7.2-7.4, 0.2 $\mu$ m sterile filtered. Stored at 4°C.

**ELISA Substrate Solution**

1:1 mixture of colour reagent A (hydrogen peroxide) and colour reagent B (tetramethylbenzidine) (R&D Systems, Abingdon, UK). Stored at 4°C.

**Stop Solution**

2N H<sub>2</sub>SO<sub>4</sub>

**2.1.5 Immunohistochemistry Reagents**

The following reagents were used in the immunohistochemistry experiments employed to evaluate tissue and cellular protein expression:

**Antigen Retrieval Solution**

5mls Vector antigen retrieval solution (Vector Laboratories, Peterborough, England) in 500mls deionised water. Freshly made for each experiment.

**Endogenous Peroxidase Block**

18mls 30% hydrogen peroxide (H-1009) in 375mls tap water. Freshly made for each experiment.

**Phosphate Buffered Saline (PBS)**

PBS tablets (Oxoid BR14) diluted in distilled water (1 tablet/100mls). Stored at room temperature.

### **2.1.6 Molecular Biology Reagents**

The following reagents were used in the molecular biology experiments employed to evaluate tissue and cellular mRNA expression:

#### **6x PCR loading buffer**

0.25% weight/volume bromophenol blue and 40% weight/volume of sucrose in distilled water. Stored at 4°C.

#### **TE Buffer**

10mM Tris-HCl (pH 8), 1mM EDTA

### **2.1.7 Protein Analysis Reagents**

The following reagents were used in Western blotting experiments employed to determine tissue protein expression:

#### **4 x Buffer pH 8.8**

1.5M Tris HCl, 0.4% SDS in distilled water. Stored at room temperature.

#### **4 x Buffer pH 6.8**

0.5M Tris HCl, 0.4% SDS in distilled water. Stored at room temperature.

#### **10% Separating Gel**

10% Polyacrylamide, 25% 4x Buffer pH8.8, 0.05% APS (A-3678), 0.05% Temed (T-9281) in distilled water. Freshly made for each experiment.

#### **4% Stacking Gel**

4% Polyacrylamide, 25% 4 x Buffer pH6.8, 0.05% APS (A-3678), 0.1% Temed (T-9281) in distilled water. Freshly made for each experiment.

**Protein Loading Buffer**

50mM Tris HCl (pH6.8), 100mM Dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol in distilled water. Stored at  $-20^{\circ}\text{C}$ .

**Protein Lysis Buffer**

50mM Tris HCl pH7.5, 320mM sucrose, 1mM EDTA (pH8), 1% protease inhibitor cocktail (P-2714), 1mM PMSF (P-7626) in 100mls distilled water. Stored at  $-20^{\circ}$ .

**5 x Running Buffer**

72g glycine (G-7403), 15.1g Trizma base (T-1503), 50mls 10% SDS, made up to 1000mls with distilled water. Diluted in distilled water to 1 x Running Buffer made up fresh for each experiment.

**Transfer Buffer**

14.4g glycine (G-7403), 2.93g Trizma base (T-1503), 200ml methanol (M-1770), made up to 1000mls with distilled water. Made up fresh for each experiment.

**Blocking Buffer**

5% Marvel in PBS with 0.05% Tween-20 (P-7949). Made up fresh for each experiment.

## 2.2 Methods

### 2.2.1 Macrophage experiments using human peripheral blood monocyte-derived macrophages

#### 2.2.1.1 Isolation of peripheral blood mononuclear cells (PBMCs) for monocyte isolation

The method used for isolation of monocytes is based on methods previously described<sup>20,420,421</sup>. Single donor Buffy coats from normal human donors were obtained from Scottish National Blood Transfusion Service. Each Buffy coat was made up to 200mls with phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugal separation over Histopaque 1077 (Sigma) for 25 minutes at 1000g. Red cells and debris collected at the base of the tube and the PBMCs appeared as a distinct creamy layer at the interface between the Histopaque and serum. The PBMCs at the interface were removed and washed in PBS, centrifuged for 10 minutes at 300g and then resuspended in PBS. In cases where a distinct creamy layer failed to form and was clearly contaminated by red blood cells, the cells at the interface were removed, washed with PBS, pelleted and resuspended in 100mls PBS. This was layered onto Histopaque for a second time. PBMCs were removed from the interface and washed twice with PBS to remove platelets.

#### 2.2.1.2 Macrophage purification and culture

CD14 positive monocytes were isolated from PBMCs by positive selection using magnetic cell sorting. This technique involves the labelling of PBMCs with magnetic CD14 microbeads, passing them through a column which is placed in the magnetic field of a VarioMACS separator. The magnetically labelled CD14+ cells are retained in the column and other cells pass through as the negative fraction. The CD14+ cells are then be eluted from the column. The procedure was performed according to the manufacturer's instruction (Miltenyi Biotech) incorporating a red cell lysis step. Briefly, 1ml of red cell lysis buffer and 4mls PBS were added to  $10^8$  PBMCs. The

cells were incubated on ice for 2 minutes. This was followed by 3 washes with PBS. The viable cells were counted using a haematocytometer with 0.4% trypan blue exclusion. Viability of the cells was always >95%.  $20 \times 10^7$  viable cells were then resuspended in 1600 $\mu$ l MACS buffer. 400 $\mu$ l CD14 MicroBeads (Miltenyi Biotec) were added and, following thorough mixing, the cells were incubated on ice for 15 minutes. The cells were then washed with MACS buffer, and the cell pellet resuspended in 500 $\mu$ l MACS buffer. The MS<sup>+</sup>/RS<sup>+</sup> columns (Miltenyi Biotec), applicable for the separation of up to  $10^7$  positive cells, were used. Prior to use, the column was washed with 500 $\mu$ l MACS buffer and the cell suspension applied. Negative cells were rinsed off the column using 3x 500 $\mu$ l buffer. The column was withdrawn from the magnetic field and the positive cells flushed out with 1ml buffer using the plunger provided. Purity checks were performed on the cells using flow cytometry and the cells were found to be consistently >93% CD14<sup>+</sup>.

Following isolation of the monocytes in this way, the cells were incubated for 1 hour at  $10^6$  cells/ml (1ml per well in 24 well tissue culture plates) in serum-free IMDM at 37°C in a humidified 5% carbon dioxide incubator to allow the monocytes to adhere to the plastic. At the end of this time, the monolayers were washed once with IMDM. Fresh IMDM-FCS was applied and the cells returned to the incubator. The cells were washed the following day and every second day thereafter. Adherence continued for 6 days.

#### 2.2.1.3 Macrophage stimulation experiments

Day 6 macrophages seeded at  $10^6$  cells per well in 24 well tissue culture plates were washed with IMDM-FCS. Where the cells were pre-incubated either with cyclopamine (Toronto Research Chemicals, Toronto, Canada), polymyxin B (PMB) or the purified CD14 functional blocking antibody 61D3 (eBioscience, San Diego, USA) or its isotype control (functional grade purified mouse IgG1 isotype (eBioscience)), this was performed for 45 minutes at 37°C prior to the addition of rShh. Recombinant murine Shh peptide was purchased from R&D Systems (Abingdon, UK) and reconstituted in IMDM-FCS to allow a final concentration range of rShh 10-1000ng/ml. Macrophages were cultured in this way for 24 hours at



37°C. At the end of this incubation period, the medium from each culture well was removed and set aside for later cytokine analysis. The cells were washed using PBS and RNA extracted where required as described in Section 2.2.5.1.

## **2.2.2 T cell experiments using human peripheral blood-derived T cells**

### **2.2.2.1 Isolation of peripheral blood mononuclear cells (PBMCs) for CD4+ T cell isolation**

As above, PBMCs were isolated from normal, single donor Buffy coats obtained from the Scottish National Blood Transfusion Service, Edinburgh. Each Buffy coat was made up to 200mls using PBS and PBMCs were obtained by centrifugation over Lymphoprep (Gibco) for 25 minutes at 1000g. The PBMCs, appearing at the interface between the Lymphoprep and serum, were removed and washed twice in PBS to remove platelets. The PBMCs were reconstituted in serum-free RPMI and plated into culture flasks at  $5 \times 10^6/\text{ml}$ . The cells were incubated for 1 hour at 37°C in a humidified 5% carbon dioxide incubator to allow adherent monocytes to be depleted from the PBMCs, by adherence to the plastic culture flask. Non-adherent cells were removed, counted and washed in PBS prior to proceeding to cell separation.

### **2.2.2.2 CD4+ T cell purification and culture**

3 methods were used to purify CD4+ T cells:

#### **2.2.2.2.1 *Preparation of CD4+ T cells using depletion columns***

PBMCs derived from centrifugation over Lymphoprep were prepared as described above. Red blood cell lysis was performed and the cells counted using a haematocytometer with 0.4% trypan blue exclusion prior to CD4 T cell separation. The Human T cell CD4 subset depletion column kit (R&D Systems) was used to isolate CD4+ cells by negative selection according to the manufacturer's instructions.

Briefly,  $2 \times 10^8$  PBMCs suspended in 1ml of column buffer (R&D Systems) were incubated with a monoclonal antibody cocktail at room temperature for 15 minutes. The cocktail binds B cells, monocytes and non-selected T cells. The cells were washed to remove any unbound antibodies and the pellet resuspended in 2mls column buffer. The column was washed with 10mls column buffer and the cell suspension applied to the column. The cells were incubated on the column for 10 minutes at room temperature and the negative fraction containing the unbound cells eluted from the column using 12mls column buffer. The cells were washed and counted. Yields of cells in the negative fraction were typically in the order of  $1.5 - 3 \times 10^7$  cells from a starting population of  $2 \times 10^8$  PBMCs. The purity of the CD4<sup>+</sup> T cells was checked using flow cytometry and the cells were found to be consistently 82-88% CD3<sup>+</sup> CD4<sup>+</sup>.

#### *2.2.2.2.2 Preparation of CD4<sup>+</sup> T cells by positive selection*

PBMCs derived from centrifugation over Lymphoprep were prepared as described above. Red blood cell lysis was performed and the viable cells counted using a haematocytometer with 0.4% trypan blue exclusion prior to CD4 T cell separation. CD4 positive cells were isolated from the PBMCs by positive selection using magnetic cell sorting. The PBMCs were labelled with magnetic CD4 microbeads (Miltenyi) and passed over a column in the magnetic field of the VarioMACS separator. The CD4<sup>+</sup> cells are retained in the column and other cells pass through as the negative fraction. The CD4<sup>+</sup> cells are then be eluted from the column. The procedure was performed according to the manufacturer's instruction (Miltenyi Biotec).  $20 \times 10^7$  cells were resuspended in 1600µl MACS buffer. 400µl CD14 MicroBeads were added and, following thorough mixing, the cells were incubated on ice for 15 minutes. The cells were then washed with MACS buffer, and the cell pellet resuspended in 500µl MACS buffer. The MS<sup>+</sup>/RS<sup>+</sup> columns (Miltenyi Biotec), applicable for the separation of up to  $10^7$  positive cells, were used. Prior to use, the column was washed with 500µl MACS buffer and the cell suspension applied. Negative cells were rinsed off the column using 3x 500µl buffer. The column was withdrawn from the magnetic field and the positive cells flushed out with 1ml buffer using the plunger provided. Yields of cells in the positive fraction were typically in

the order of  $2.5 - 3.8 \times 10^7$  cells from a starting population of  $2 \times 10^8$  PBMCs. Purity checks were performed on the cells using flow cytometry and the cells were found to be consistently >95% CD3+ CD4+.

#### *2.2.2.2.3 Preparation of CD4+ T cells by FACS cell sorting*

PBMCs derived from centrifugation over Lymphoprep were prepared as described above. Red blood cell lysis was performed and the cells counted using a haematocytometer with 0.4% trypan blue exclusion prior to CD4 T cell separation. The Human T cell CD4 subset column kit (R&D Systems) was used to isolate CD4+ cells by negative selection as described in 2.2.2.2.1 above. The cells produced were then incubated with an excess ( $1\mu\text{g}/10^6$  cells) of APC-labelled mouse anti-human CD4+ and PE-labelled mouse anti-human CD3+ antibodies and incubated at 4°C for 25 minutes to allow labelling of the cells to occur. The cells were then washed in FACS buffer for 5 minutes at 100g and the cell pellet was reconstituted in 1% serum-containing RPMI at a concentration of  $10^7$  cell/ml. FACS cell sorting was performed by Shonna McCall (flow cytometry technician, University of Edinburgh) using a FACSVantage (BD Biosciences, San Jose, CA, USA) sorter. CD3+ CD4+ T cells were sorted into medium containing 5% AB serum and the cells were used for culture as above. From a starting population of negatively selected cells, yields of CD3+ CD4+ T cells were typically in the order of 20-25%. Cells purified in this way were typically >98.8% pure.

#### 2.2.2.3 CD4+ T cell stimulations using anti-CD3 $\epsilon$ and anti-CD28 antibodies

CD4+ T cell stimulations were performed using plate-bound anti-CD3 $\epsilon$  and soluble anti-CD28 antibodies (BD Pharmingen, San Diego, CA, USA) as described previously<sup>20,21</sup>. Briefly, the anti-CD3 $\epsilon$  antibody was first immobilised onto the wells of 96 well round bottomed plates by diluting the antibody in PBS to final concentrations of either  $0.2\mu\text{g}/\text{ml}$  or  $1\mu\text{g}/\text{ml}$ . The plates were incubated for 2 hours at 37°C and then washed 3 times with PBS to remove unbound antibody. The plates were air-dried for 30 minutes at 37°C prior to use. Anti-CD28 antibody was reconstituted in medium to final concentrations of  $1\mu\text{g}/\text{ml}$  and  $5\mu\text{g}/\text{ml}$ . Thus, anti-

CD3 $\epsilon$  at 0.2 $\mu$ g/ml with anti-CD28 at 1 $\mu$ g/ml constituted a “suboptimal” stimulus and anti-CD3 $\epsilon$  at 1 $\mu$ g/ml with anti-CD28 at 5 $\mu$ g/ml constituted an “optimal” stimulus in keeping with previous studies<sup>20,21</sup>. Each condition was replicated in quadruplicate on the 96-well plate. The T cells were cultured in these conditions for 72 hours.

#### 2.2.2.4 Mixed leukocyte reactions (MLRs)

Macrophages were prepared by maturing CD14<sup>+</sup> PBMC-derived monocytes as described in Section 2.2.1.2 for 6 days in 162cm<sup>2</sup> tissue culture flasks (Costar, Fisher Scientific, Loughborough, Leicestershire, UK). Following this incubation, the cells were removed from the culture flasks using cell lifters (Costar). The macrophages were treated with Mitomycin-C (MMC)(Sigma), an antibiotic that inhibits nucleic acid synthesis. The cells were mixed with 50 $\mu$ g/ml MMC and incubated for 30 minutes at 37°C. The cells were then thoroughly washed 4 times with ice-cold medium to remove any residual MMC. Cells prepared in this way were counted and plated out into round-bottomed 96 well culture plates at a range of concentrations from 10<sup>4</sup> - 5 x 10<sup>5</sup> cells per well. These cells were used to stimulate CD4<sup>+</sup> T cells from a different donor in the mixed leukocyte reactions. The MLRs were incubated for 72 hours.

#### 2.2.2.5 Proliferation assay

After 72 hours in culture, proliferation was evaluated by pulsing the cells with tritiated methyl thymidine (<sup>3</sup>H-TdR-Amersham Pharmacia Biotech, Amersham, UK) at a concentration of 1 $\mu$ Ci/well and incubating for a further 18 hours. <sup>3</sup>H-TdR incorporation was evaluated using a Betaplate 1205 liquid scintillation counter (Wallac, Milton Keynes, UK).

## **2.2.3 Renal Epithelial Experiments**

### **2.2.3.1 ACHN Cell Line Management**

An aliquot of the ACHN human renal adenocarcinoma cell line (ECACC No. 88100508) was a gift from Dr Ewan Harrison (Centre for Inflammation Research, University of Edinburgh). Cells were grown in complete DMEM. The ACHN cells were grown in culture flasks and incubated in 5% CO<sub>2</sub> at 37°C. When the cells reached ~75% confluence, the cells were incubated in 0.5g/L trypsin/0.2g/L EDTA (Invitrogen, Paisley, UK) for 10 minutes at 37°C to lift the cells from the base of the flask. The cells were washed in complete DMEM and centrifuged at 300g for 5 minutes. The cell pellet was reconstituted in complete DMEM and the cells split 1:5. Where cells were to be set aside for storage, 5 x 10<sup>6</sup> cells were resuspended rapidly in 1ml of freezing mix and stored at -70°C for 2 days. The cells were subsequently transferred to liquid nitrogen for long-term storage.

### **2.2.3.2 Murine primary renal epithelial cell (REC) culture**

Normal kidneys were removed from C57BL/6 mice and placed whole into complete HBSS. The kidneys were rinsed in HBSS and the capsule and pelvis removed by sharp dissection. The kidneys were placed in a petri dish, chopped into small pieces ~1mm<sup>3</sup> in size and transferred to a universal container. 10mls of collagenase mixture were added, mixed with the kidney pieces and incubated at 37°C for 30 minutes. The mixture was agitated every 10 minutes to aid digestion. After 30 minutes, the mixture was agitated and the pieces allowed to settle. A sample of supernatant was taken at this time and observed under the microscope. If short tubules were present, the supernatant was removed and placed in a clean universal container with fresh HBSS. The tubules were settled out by placing the mixture on ice for 20 minutes. After 20 minutes, the supernatant was removed and replaced with fresh HBSS. After a further 20 minute incubation on ice, the tubules were resuspended in complete DMEM F12 culture medium and plated out into flasks. The medium was changed every 2-3 days until confluence was achieved (no more than 10 days).

## **2.2.4 Protein Analysis**

### **2.2.4.1 Flow Cytometry**

Flow cytometry was used to analyse the expression of cell surface molecules. Briefly,  $2-3 \times 10^5$  cells were washed with 500µl FACS wash and centrifuged at 4°C for 5 minutes at 300g. The cells were then incubated in 50µl FACS wash buffer and 200ng of the relevant directly conjugated monoclonal antibody (BD Pharmingen, San Diego, CA, USA) in the dark at 4°C for 25 minutes. The antibodies used for flow cytometry were Phycoerythrin (PE) – Cy5 mouse anti-human CD14 antibody and mouse IgG<sub>2a</sub> isotype control (Serotec, Kidlington, Oxford, UK), PE mouse anti-human CD3 and mouse IgG<sub>1</sub>κ (BD Pharmingen, San Diego, CA, USA), Allophycocyanin (APC) mouse anti-human CD4 and mouse IgG<sub>1</sub>κ isotype (both BD Pharmingen, San Diego, CA, USA). The cells were washed with 500µl FACS wash. Where immediate analysis of the samples took place, the cell pellet was resuspended in 300µl FACS buffer and analysed. Where sample analysis was delayed, the cell pellet was instead reconstituted in 300µl FACS wash buffer together with 200µl FACS fixing reagent. Fixed samples were stored for no longer than 1 week in the dark at 4°C. Sample acquisition was performed using a Becton Dickinson FACSCalibur (BD Immunocytometry Systems, San Jose, CA, USA) and analysed using BD CellQuest Software with help from Shonna McCall (flow cytometry technician, University of Edinburgh).

### **2.2.4.2 Enzyme Linked Immunosorbent Assay (ELISA)**

Duoset ELISAs were used to quantify human TGF-β1 and VEGF and murine Shh protein in cell culture supernatants, according to the manufacturer's instructions (R&D Systems, Abingdon, Oxfordshire, UK). Both latent and active TGF-β1 were assayed using the Duoset TGF-β1 ELISA. To evaluate latent TGF-β1, activation was performed using 1N HCl followed by 1.2N NaOH/0.5M HEPES according to the manufacturer's instructions. The resulting product was then analysed in parallel with



the untreated samples on the TGF- $\beta$  ELISA. Briefly, for all ELISAs, the appropriate capture antibody was diluted in PBS and used to coat the wells of 96 well immunoassay plates (Corning, NY, USA). The plates were sealed and incubated overnight at room temperature. The capture antibody was removed and the wells washed 3 times using ELISA wash buffer. 300 $\mu$ l of the appropriate block buffer was applied to each well for at least 1 hour at room temperature. The block buffer was removed and the wells washed 3 times with ELISA wash buffer. 50 $\mu$ l of either standard or sample was applied to well. Both standards and samples were run in duplicate. The plates were sealed and incubated at room temperature for 2 hours. Wells were washed 3 times with ELISA wash buffer and the detection antibody diluted in ELISA reagent diluent, applied in a volume of 50 $\mu$ l per well. Following a 2 hour incubation, the detection antibody was removed and the wells again washed with ELISA wash buffer. 50 $\mu$ l streptavidin-HRP was applied to each well. The plate was covered and incubated for 20 minutes at room temperature. Further washing with ELISA wash buffer was followed by the addition of 50 $\mu$ l substrate solution and, once again, the plates were protected from light. After 20 minutes, the reaction was stopped using 25 $\mu$ l stop solution. The optical density of each well was evaluated using a microplate reader (Dynatech Laboratories, USA) set to a wavelength of 450nm with wavelength correction set to 570nm.

#### 2.2.4.3 Cytometric Bead Arrays (CBA)

The Cytometric Bead Array (CBA) (BD Biosciences, San Diego, USA) allows the simultaneous flow cytometric evaluation of several cytokines in a single small sample (50 $\mu$ l) of supernatant. The assay utilises bead populations and each population is coated with antibodies specific for a cytokine. The bead populations each have distinct fluorescence intensities resolved in the FL3 channel of the flow cytometer. The CBAs were performed according to the manufacturer's instructions. The CBAs used in this thesis and the cytokines analysed by each CBA are shown in Table 1. The standard cocktail was reconstituted in 200 $\mu$ l reagent diluent and then a standard curve was generated by serial dilutions of the top standard in reagent diluent (BD Biosciences). 50 $\mu$ l of either standard or vortexed sample was placed in 75mm



tubes (BD Falcon). 5µl of each cytokine bead suspension were mixed together by gentle vortexing and 25µl of the bead mix was added to the 50µl of standard or sample already present. To each tube, 25µl PE detection reagent (BD Biosciences) was added and the contents of each tube were mixed by brief vortexing. The samples were protected from light and incubated for 3 hours at room temperature to allow sandwich complexes to form. At the end of this incubation, the beads were washed with 500µl wash buffer (BD Biosciences) and centrifuged for 5 minutes at 300g. The supernatant was discarded and, following resuspension of the beads in 200µl wash buffer, the data was acquired using a Becton Dickinson FACSCalibur (BD Immunocytometry Systems) flow cytometer. The analysis was performed using the BD CBA analysis software. The assays were sensitive within the range 20-5000pg/ml. Where values over 5000pg/ml were obtained, the assay was repeated following further dilution of the samples.

**TABLE 1: Cytometric Bead Arrays (CBAs)**

Table lists CBAs used in thesis and the cytokines or chemokines analysed by each kit.

Cytometric Bead Array	Cytokines analysed
Human Inflammation	IL-1 $\beta$ , TNF- $\alpha$ , IL-10, IL-6, IL-12p70, IL-8
Human Chemokine	IP-10, MCP-1, MIG, RANTES, IL-8
Human Th1/Th2	IFN- $\gamma$ , TNF- $\alpha$ , IL-10, IL-5, IL-4, IL-2

#### 2.2.4.4 Immunohistochemistry

##### 2.2.4.4.1 *Tissue Specimens*

Ethical approval was obtained from Lothian Research Ethics Committee. Normal human renal tissue was obtained from 3 cadaveric transplant donors whose kidneys were not placed for transplantation. Prior consent to the use of these kidneys for research purposes was obtained from the donors' relatives. Further fresh tissue was obtained from 3 patients undergoing transplant nephrectomy for CAN. A further 10 specimens of normal and CAN kidney were obtained from the pathology archives.

##### 2.2.4.4.2 *Immunohistochemistry using formalin-fixed, paraffin-embedded (FFPE) tissue sections*

Immunohistochemistry was used to determine the tissue expression of proteins. Three  $\mu\text{m}$  sections were cut from archival formalin-fixed, paraffin-embedded (FFPE) tissue and mounted on Vectabond treated slides (Vector Laboratories, Peterborough, England). The paraffin sections were de-waxed in xylene and rehydrated through descending alcohols. Antigen retrieval was performed using Vector antigen retrieval solution (Vector Laboratories) according to the manufacturer's instructions. Following a wash in PBS, endogenous peroxide in the tissue sections was blocked using 3% hydrogen peroxide for 10 mins and the slides were loaded onto Sequenza clips (Shandon Scientific Ltd, Cheshire, UK). Avidin/biotin blocking (Avidin/Biotin blocking kit, Vector Laboratories) was performed on all slides according to the manufacturer's instructions and non-specific binding was blocked using Dako serum-free protein block (Dako Ltd, Ely, UK). The sections were probed with the appropriate primary antibody for either 1-2 hours at room temperature or overnight at 4°C depending on the antibody used. The primary antibodies used included: goat polyclonal antibodies to N-terminus of Shh and C-terminus of Ptc (at 1:40 and 1:30 dilution respectively)(Santa Cruz, Biotechnology), mouse monoclonal anti-CD68 (at 1:20 dilution)(Dako Ltd), mouse monoclonal anti-CD3 (at 1:30 dilution), chicken polyclonal anti-TGF- $\beta$  (1:50 dilution) (R&D Systems, Abingdon, UK) and rabbit polyclonal anti-VEGF (1:40 dilution) (Santa Cruz Biotechnology). To check

specificity, the polyclonal Santa Cruz antibodies were preincubated overnight with the appropriate specific blocking peptide, where available, according to the manufacturer's instructions (Santa Cruz Biotechnology). These blocking peptides are designed to bind to the epitope of the antibody responsible for binding to the protein of interest and, thus, when used in excess, should not allow binding of the antibody to the specific protein to occur. In accordance with the manufacturer's instructions (Santa Cruz Biotechnology), the antibodies were each pre-incubated with a five-fold excess (by weight) of blocking peptide and then applied to the sections in experiments performed in parallel with experiments in which primary antibodies were applied to the slides. Following the overnight incubation, the slides were washed with PBS and the appropriate biotinylated secondary antibody applied (Dako Ltd, Ely, UK) at 1:400 dilution for 30 mins. Further washing was followed by the application of Vector RTU ABC (Vector Laboratories, Peterborough, UK) and positive signalling identified using diaminobenzidine (Dako Ltd). The slides were finally counterstained with haematoxylin.

#### 2.2.4.4.3 *Picrosirius red staining*

Picrosirius red is a collagen stain that appears as a red stain, but under polarised light appears green<sup>422</sup>. Dewaxing of the paraffin sections and rehydration through descending alcohols was followed by antigen retrieval using Vector antigen retrieval solution (Vector Laboratories) according to the manufacturer's instructions. Following a wash in PBS, the sections were stained for 10 minutes in Picrosirius red (0.1% Sirius red F3BA in picric acid) (Sigma) and then washed again in PBS prior to rehydration through alcohol and mounting.

#### 2.2.4.4.4 *Macrophage Immunohistochemistry*

CD14 positive monocytes were isolated from human PBMCs by positive selection using magnetic cell sorting as outlined in section 2.2.1.2 above. The cells were counted using a haemocytometer with 0.4% trypan blue exclusion. Glass coverslips were rinsed in alcohol and allowed to air dry. Once dry, the coverslips were placed at

the base of 6 well tissue culture plates. Monocytes were plated into each well at a concentration of  $10^6$ /ml with 2mls per well in serum-free medium (IMDM). Following a 1 hour adherence step, the medium was then replaced with IMDM-FCS and the macrophages were allowed to mature over the course of 6 days with a change of medium every 2 days. After 6 days, the medium was removed and the macrophages were washed thoroughly 3 times with PBS. The cells were fixed using a 10% dry acetone/90% methanol mix for 5 minutes. The coverslips were then allowed to air dry. A further PBS wash was followed by blockade of the endogenous peroxidase for 10 minutes using hydrogen peroxide as above. The primary antibodies were then applied to the coverslips in a final volume of 300 $\mu$ l and left overnight at 4°C. The primary antibodies used for the macrophage experiments were goat polyclonal antibodies to the N-terminus of Shh (1:40 dilution), C-terminus of Ptc (1:30 dilution), Smo (1:30 dilution) and Gli-1 (1:30 dilution) (Santa Cruz Biotechnology, Insight Biotechnology Ltd, Wembley, Middlesex, UK). All antibodies were diluted in Dako diluent immediately prior to use. To check antibody specificity, a further aliquot of each antibody was preincubated overnight with its specific blocking peptide. Cells treated in this way served as negative controls to the experiments. Following overnight incubation, three 5 minute PBS washes were performed and then biotinylated anti-goat IgG secondary antibody (Dako Ltd, Ely, UK) diluted 1:400 in Dako diluent was applied to the coverslips for 1 hour. This was followed by 3 further PBS washes and the application of Vector RTU ABC (Vector Laboratories, Peterborough, UK) for 30 minutes. Positive signalling was identified using diaminobenzidine (Dako Ltd). The coverslips were counterstained with haematoxylin prior to mounting.

#### 2.2.4.4.5 *ACHN and REC Immunohistochemistry*

ACHN or REC cells were grown to approximately 75% confluence on glass coverslips placed at the bases of 6 well culture plates. The medium was removed and the cells washed thoroughly 3 times with PBS taking care not to lift the cells from the coverslips during washing. The cells were fixed using a 10% dry acetone/90% methanol mix for 5 minutes. The coverslips were allowed to air dry. The primary

antibodies were then applied to the coverslips in a final volume of 300µl and left overnight at 4°C. The primary antibodies used for the ACHN cells were goat polyclonal antibodies to the N-terminus of Shh (1:40 dilution) and C-terminus of Ptc (1:30 dilution) (Santa Cruz Biotechnology, Insight Biotechnology Ltd, Wembley, Middlesex, UK). The same antibodies were used with the RECs together with monoclonal anti-pancytokeratin and -vimentin antibodies (Sigma) used at 1:100 dilution. All antibodies were diluted in Dako diluent immediately prior to use. To check the antibody specificity of the anti-Shh and anti-Ptc antibodies, a further aliquot of each antibody was preincubated overnight with its specific blocking peptide where available. This was then applied to the coverslips in experiments performed in parallel with experiments in which primary antibodies were applied to the coverslips. The cells were incubated for 2 hours at room temperature with the anti-pancytokeratin and anti-vimentin antibodies and overnight at 4°C with the anti-Shh and anti-Ptc antibodies. For the anti-pancytokeratin and anti-vimentin antibodies, omission of the primary antibody served as the negative control. The primary antibody incubations were followed by three 5 minute PBS washes and the appropriate biotinylated secondary antibody, either rabbit anti-goat or rabbit anti-mouse antibodies (Dako Ltd, Ely, UK) diluted 1:400 in Dako diluent was applied to the coverslips for 1 hour. This was followed by 3 further PBS washes and the application of Vector RTU ABC (Vector Laboratories, Peterborough, UK) for 30 minutes. Positive signalling was identified using diaminobenzidine (Dako Ltd). The coverslips were counterstained with haematoxylin prior to mounting.

#### 2.2.4.5 Western Blotting

##### 2.2.4.5.1 *Protein Lysis*

Adherent cells were removed from the bases of tissue culture wells using a cell scraper and immediately centrifuged with ice cold PBS at 300g for 5 minutes at 4°C. The supernatant was removed and protein lysis buffer applied to the cell pellet. For the renal epithelial cell experiments, 200µl protein lysis buffer for the 75% confluent cells in 3 wells of a 6 well culture plate was used. The lysates were mixed thoroughly on ice and sonicated for 30 seconds in 5 minute bursts prior to protein quantification.

##### 2.2.4.5.2 *Protein Quantification*

Protein was quantified using the Bio-Rad (Bradford) protein assay (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) according to the manufacturer's instructions. Briefly, the Bio-Rad reagent was diluted 1:5 with distilled water and filtered into a foil-covered storage vessel through Whatman Grade No. 1 filter paper. A 10mg/ml bovine serum albumin (BSA) stock solution was prepared using distilled water and this was used to generate a standard curve of protein concentrations diluted to the appropriate concentration using protein lysis buffer. 2 mls of freshly prepared Bio-Rad reagent were added to a cuvette. 50µl of standard or sample was added to the cuvette, mixed with the Bio-Rad reagent and incubated at room temperature for 15-20 minutes. After this incubation, the samples were read on the spectrophotometer at 595nm (OD<sub>595</sub>). All samples were run in triplicate and the values were normalised back to the negative control (Bio-Rad reagent and 50µl distilled water). The values for the standards were used to generate a standard curve from which the sample values were extrapolated.

##### 2.2.4.5.3 *Western Blot Protocol*

Western blots were performed using cellular protein, lysed and quantified as described above. The technique was also used in an attempt to detect protein in cell



culture supernatants from cells grown in 10mls medium in 162cm<sup>2</sup> culture flasks. In this instance, each supernatant was transferred to dialysis tubing and placed on polyethylene glycol (PEG) (Fisher Scientific UK, Loughborough, Leics, UK), the latter binding water and thus concentrating the protein to a volume of less than 2.5mls. The protein content was quantified, as previously, using the Bradford protein assay, performed on each sample in triplicate. 100µg of protein was then used for the Western blot experiments.

10% polyacrylamide electrophoresis gels were cast and overlaid with 4% stacking gels. 100µg protein samples were added to 2x protein loading buffer, heated at 100°C for 10 minutes, and electrophoresed at 100V for 2 hours in 1x running buffer alongside pre-stained molecular weight markers (Invitrogen, Paisley, UK). The electrophoresed samples were then transferred to nitrocellulose in transfer buffer at 150V for 30 minutes. The membranes were washed in ELISA wash buffer and then incubated overnight in blocking buffer to block non-specific binding. The membranes were washed 3 times using ELISA wash buffer and then probed for 2 hours with the appropriate antibody diluted in 2.5% Marvel in PBS with 0.05% Tween-20. In parallel experiments, to confirm antibody specificity, an aliquot of the antibody was pre-incubated with the specific blocking peptide (Santa Cruz Biotechnology), where available, prior to application to the membrane. Following incubation with the primary antibody, the membranes were washed 3 times with ELISA wash buffer. The secondary antibodies were made up to the appropriate dilution in ELISA wash buffer and were applied for 2 hours. The membranes were washed a further 3 times. Detection was effected using the ECL plus system (Amersham Pharmacia Biotech, Amersham, UK) according to the manufacturer's instructions. Briefly, 2000µls solution A was added to 50µl solution B, mixed and applied to the membrane for 5 minutes. Excess reagent was removed and the membrane placed under X-ray sensitive film for various lengths of exposure time. The films were processed using an X-Ograph X-ray developer (X-Ograph Imaging Systems, Wilts, UK).

## 2.2.5 Molecular Biology

### 2.2.5.1 RNA Extraction

Where RNA was required from fresh renal tissue, 1mm<sup>3</sup> cubes of tissue were prepared using sharp dissection. The tissue was homogenised in Trizol (Gibco) such that the tissue volume did not exceed 10% of the volume of Trizol. Where RNA was required from cells grown in tissue culture wells, 1ml Trizol was applied to 5-10 x 10<sup>6</sup> cells. The samples were incubated in Trizol for 5 minutes at room temperature. Where immediate RNA extraction was not performed, the samples in Trizol were transferred to storage at -70°C for no longer than 4 weeks. Following the homogenisation step, per 1 ml Trizol, 200µl chloroform was added and the mixture vigorously shaken for 15 seconds. Following a 5 minute room temperature incubation, the mixture was centrifuged at 12,000g for 15 minutes at 4°C. The upper aqueous phase was carefully removed and transferred to a 1.5ml RNase-free eppendorf. To this was added 500µl of isopropyl alcohol. The mixture was inverted a few times, incubated for 10 minutes at room temperature and centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was removed, washed with 75% ethanol and vortexed. The sample was centrifuged at 9500g for 5 minutes at 4°C and the supernatant removed. The RNA pellet was then air-dried by inverting the open eppendorf onto clean tissue paper and leaving for 30 minutes at room temperature. At the end of this time, the RNA pellet was dissolved in 10µl RNase-free water.

#### 2.2.5.1.1 *DNase Treatment of RNA*

Contaminating DNA was removed from the RNA using the RNase-free DNase set (Gibco) according to the manufacturer's instructions. 1µg of RNA was added to 1µl of 10x DNase buffer 1 and DEPC water added to a final volume of 10µl. The mixture was incubated for 15 minutes at room temperature. To this mixture, 1µl of 25mM EDTA was added and incubated for 10 minutes at 65°C.

#### 2.2.5.1.2 *Quantification of RNA*

The RNA was quantified by measuring absorbance at 260 and 280nm in a spectrophotometer (Ultrospec, Pharmacia Biotech). The purity was evaluated by determining the ratio of absorbance at 260nm and 280nm (OD<sub>260</sub>/280). For pure RNA, this should be around 2.0.

#### 2.2.5.1.3 *Testing RNA Quality*

RNA quality was evaluated by reconstituting 1µg of RNA into a total volume of 10µl in RNase free water. This was mixed with 2µl 6x PCR loading buffer which was heated at 65°C for 5 minutes and cooled on ice for 3 minutes. The sample was run on a 1% agarose gel at 60 volts for 40 minutes. When viewed under a white/ultraviolet transilluminator, good quality RNA should appear as 2 discrete bands. Conversely, a smear indicates degraded or poor quality RNA.

#### 2.2.5.2 Reverse Transcription of RNA to cDNA

Reverse transcription of 400ng RNA was performed using the Taqman Reverse Transcription Reagent Kit (Applied Biosystems, Warrington, Cheshire, UK). Briefly, 400ng RNA in a maximum volume of 4µl was mixed with 2µl 10x Taqman buffer, 4.4µl MgCl<sub>2</sub>, 4µl dNTP, 1µl random hexamers, 0.4µl RNase inhibitor, 0.5µl Multiscribe reverse transcriptase and 3.7µl nuclease free water to give a final reaction volume of 20µl. Each reaction in a 200µl RNase-free tube was amplified in a PTC 200 Peltier Thermal Cycler in a programme with the following conditions: 10 minutes at 25°C, 40 minutes at 48°C and 5 minutes at 95°C. The cDNA produced was stored at -20°C.

#### 2.2.5.3 Standard PCR

500ng cDNA was amplified in each 20µl reaction. A typical PCR reaction contained 1.8µl reaction buffer (BioGene, Kimbolton, Cambridge, UK), 1.5mM MgCl<sub>2</sub> or 1.2mM Mg Cl<sub>2</sub> depending on the primer, 1µl of each primer, 2µl cDNA, 100µM

dNTPs, 0.1% bovine serum albumin, 0.25 units Taq polymerase (Biogene). The PCR cycle programme was 94°C for 3 minutes, 40 cycles of 94°C for 1 minute, annealing temperature <sup>TM</sup> specific for each gene for 1 minute (See Table 2) and, finally, 72°C for 1 minute 30 seconds. PCR products were analysed by electrophoresis using a 1% agarose gel, stained with ethidium bromide.

The primers and annealing temperatures used for each gene are shown in Table 2.

**TABLE 2: RT-PCR Primers**

Table lists primer sets used for the evaluation of Shh signalling pathway components using RT-PCR.

Gene	Primer Sequence 5'-3'	PCR product (bp)	Tm (°C)
<b>Human Shh</b>	<b>Forward:</b> act ggg tgt act acg agt cca agg <b>Reverse:</b> aaa gtg agg aag tcg ctg tag agc	211	60
<b>Human Ptc</b>	<b>Forward:</b> tcc tcg tgt gcg ctg tct tcc ttc <b>Reverse:</b> cgt cag aaa ggc caa agc aac gtg a	462	60
<b>Human Smo</b>	<b>Forward:</b> ctg gta cga gga cgt gga gg <b>Reverse:</b> agg gtg aag agc gtg cag ag	140	62
<b>Human Gli1</b>	<b>Forward:</b> act gaa gac ctc tcc agc <b>Reverse:</b> gct gac agt ata ggc aga	244	62
<b>Human Gli2</b>	<b>Forward:</b> tgg ccg ctt cag atg aca gat gtt g <b>Reverse:</b> cgt tag ccg aat gtc agc cgt gaa g	200	62
<b>Murine Shh (1)</b>	<b>Forward:</b> agg ggg ttg gga aag agg <b>Reverse:</b> gga ttc ata gta gac cca gtc g	450	60
<b>Murine Shh (2)</b>	<b>Forward:</b> tta aat gcc ttg gcc atc tc <b>Reverse:</b> cca cgg agt tct ctg ctt tc	200	60
<b>Murine Shh (3)</b>	<b>Forward:</b> atg tca gtg tca tcc gag tgg c <b>Reverse:</b> tga ata cca cca cca cag cag c	250	65
<b>β-Actin</b>	<b>Forward:</b> cca cca act ggg acg aca tg <b>Reverse:</b> gta tca aac atg atc tgg gtc atc	151	58

#### 2.2.5.4 Real Time PCR

Real time PCR is a method of quantifying PCR products as they are produced, hence the term 'real time'. The technique employs the 3'-5' exonuclease activity of *Taq* polymerase to cleave a fluorescent probe which is then detected and quantified. The probe is 20-30 oligonucleotides long and contains the reporter fluorescent dye ('fam') and a quencher dye at the 5' and 3' end respectively. Such close proximity of the reporter fluorescent dye and the quencher dye prevents fluorescent emission while the probe is intact. However, when the probe is cleaved, the quencher and reporter are separated and the fluorescent signal increases proportionally to the amount of PCR product generated. The amount of fluorescent signal generated at each cycle produces a sigmoid waveform from which it is possible to detect the first significant increase in the amount of PCR product. This correlates with the initial quantity of target template. The threshold cycle ( $C_T$ ) value is the cycle when a significant increase in reaction product is first detected. The higher the initial amount of genomic DNA, the earlier the product accumulates and the sooner it is detected by the system, giving a lower  $C_T$  value. A  $C_T$  value of 40 suggests no amplification and no significant PCR product. The fam values generated are then normalised to 18S rRNA which serves as the endogenous internal control and is labelled with the fluorescent dye 'vic'.

Primers and probes were designed using Primer Express software and the combinations used are shown in Table 3. The conditions employed for all reactions were: 2 minutes at 50°C, 10 minutes at 95°C, 15 seconds at 95°C and 1 minute at 60°C and this cycled 40 times. Each sample was run in triplicate and each set of samples was run against its own control i.e. treated versus untreated where the untreated samples were always given a value of 1. Results were expressed relative to the untreated sample value.

**TABLE 3: Real-Time Primer and Probe Sequences**

Table lists primer and probe sets used for evaluation of the Shh signalling pathway components using Real-Time PCR

Gene	Primer Sequence (5'-3')
<b>Human Shh</b>	<b>Forward:</b> taa gga caa gtt gaa cgc ttt gg <b>Reverse:</b> tcg gtc acc cgc agt ttc <b>Probe:</b> cat ctc ggt gat gaa cca gtg gcc a
<b>Human Ptc</b>	<b>Forward:</b> cgg cag ccg cga taa g <b>Reverse:</b> tta atg atg cca tct gca tcc a <b>Probe:</b> atc gac atc agc cag ttg act aaa cag cgt c
<b>Murine Shh (1)</b>	<b>Forward:</b> tga ccc ctt tag cct aca agc a <b>Reverse:</b> ttc ttg tga tct tcc ctt cat ata tct g <b>Probe:</b> ttt att ccc aac gta gcc gag aag acc c
<b>Murine Shh (2)</b>	<b>Forward:</b> tga ccc ctt tag cct aca agc a <b>Reverse:</b> ttt ttg tga tct tcc ctt cat atc tg <b>Probe:</b> ttt att ccc aac gta gcc gag aag acc c
<b>Murine Shh (3)</b>	<b>Forward:</b> gag cag acc ggc tga tga ct <b>Reverse:</b> aga gat ggc caa ggc att taa ct <b>Probe:</b> tgt ctt tgc acc tct g
<b>Murine Ptc</b>	<b>Forward:</b> ctc caa gtg teg tcc ggt tt <b>Reverse:</b> tgt act ccg agt cgg agg aat c <b>Probe:</b> cgt gcc tcc tgg tca cac gaa caa



### 2.2.6 Endotoxin Assay

The *Limulus* Amebocyte Lysate (LAL) assay was used to evaluate the LPS content of reagents used in the *in vitro* experiments. The system used was a LAL endpoint chromogenic assay (Associates of Cape Cod, Falmouth, Massachusetts, USA). This chromogenic assay uses a modified LAL reagent (Pyrochrome) incorporating an aqueous extract of the amebocytes of *Limulus polyphemus* and a chromogenic substrate<sup>423</sup>, cleavage of which releases the yellow substance p-nitroaniline which absorbs at 405nm. The amount of p-nitroaniline released is assayed after a fixed incubation time. The assay was performed according to the manufacturer's instructions. Briefly, the control standard endotoxin (CSE) was reconstituted in LAL reagent water and a serial dilution was prepared to generate a set of standards. 50µl sample or standard was then added to 50µl Pyrochrome in the wells of a sterile 96 well plate and mixed thoroughly for 30 seconds on a plate-shaker. The plate was transferred to an incubator block set at 37°C. After 20 minutes, the plate was removed from the incubator block and the reaction stopped using 20µl of 50% acetic acid. The optical density was then read in a plate reader set at 405nm. All samples were prepared in quadruplicate and the mean optical density of the negative control (LAL water alone) was subtracted from all other readings. The results obtained from the samples were plotted against a standard curve consisting of measured optical density plotted against the standard endotoxin concentrations.

### 2.2.7 MTT Assay

MTT is a water soluble tetrazolium salt. When the tetrazolium ring is cleaved by dehydrogenase enzymes<sup>424</sup>, dissolved MTT is converted to an insoluble purple formazan. This can be solubilised and then measured spectrophotometrically to yield absorbance as function of concentration of converted dye. Only active mitochondrial dehydrogenases of living, and not dead, cells cause the conversion. The MTT assay was performed according to the manufacturer's instructions (Sigma M-5655). The MTT was reconstituted to 5mg/ml in PBS. A maximum of 3 hours before the end of the incubation,  $\frac{1}{10}^{\text{th}}$  of the culture medium volume was removed and replaced



with the same volume of MTT stock solution. The cells were returned to the incubator and, at the end of the incubation period, the medium was removed and the converted dye solubilised using DMSO. 100µl aliquots of each well were transferred to a 96 well plate and the absorbance read at a wavelength of 570nm.

## **Chapter 3**

**Sonic hedgehog is differentially expressed in normal kidney and chronic renal allograft nephropathy**

### 3.1 Introduction

As discussed in Chapter 1, the highly conserved hedgehog family of proteins has an important role in determining both cell fate and proliferation during development. Sonic hedgehog (Shh) acts as a mitogen, inducing proliferation in keratinocytes<sup>425</sup>, neuronal precursor cells<sup>426</sup> and haematopoietic precursors<sup>19</sup>. Expression of pathway components is widespread in the embryo and Shh is required for developmental patterning and cell fate induction in various organ systems<sup>3</sup>. In particular, Shh signalling has roles in the development of the gut<sup>4,5,7,427</sup> lungs<sup>4,5,16</sup> and central nervous system<sup>5</sup>.

Less, however, is known about the role of Shh signalling in the development of the kidneys. Shh mutants have a single fused midline kidney, and Gli2-/-;Gli3+/- mutants have paired horseshoe-shaped kidneys<sup>137</sup>. In the metanephric kidney, Shh expression has been reported in the urogenital sinus, the tip of the urethral epithelium<sup>428</sup> and in the uroepithelium of collecting ducts and the ureter<sup>2,4,138,139</sup>. There have been no previous reports of Shh expression in the adult human kidney although expression of another vertebrate Hh family member, Ihh, has been reported<sup>2,146</sup>.

Chronic renal allograft nephropathy (CAN) is the major cause of long-term renal failure in kidney allograft recipients. It is characterised by a mononuclear cell infiltration (consisting predominantly of macrophages and T cells), interstitial fibrosis, tubular atrophy and dilatation, and vascular wall thickening<sup>226,227</sup>. The progressive fibrosis that occurs is considered to be the main cause for the gradual deterioration in renal function and graft failure.

That the Shh pathway may play a role in the aetiology of CAN is suggested by several lines of evidence. Specifically, Stewart et al<sup>149</sup> demonstrated recently the upregulation of Shh in epithelial cells at sites of fibrotic disease in both human fibrotic lung disease (cryptogenic fibrosing alveolitis and bronchiectasis) and in murine models of fibrotic lung disease, suggesting that the pathway may play a role

in the development of fibrosis. Furthermore, components of the Shh pathway have been demonstrated in immune cells recognised to play a role in the pathogenesis of CAN. Both T cells and macrophages<sup>246</sup> are present in the inflammatory cell infiltrate in CAN with macrophage infiltration and activity correlating directly with graft survival<sup>241</sup>. Shh, Ptc and Gli1 expression have been demonstrated in T cells, and Ptc expression has also been reported in macrophages<sup>149</sup>. Furthermore, other factors known to be upregulated in CAN may also be upregulated by Shh. These include the growth factors, Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) and Vascular Endothelial Growth Factor (VEGF).

TGF- $\beta$  stimulates fibroblasts to synthesise matrix proteins, including collagen, fibronectin and proteoglycans<sup>274</sup> and inhibits the degradation of newly formed matrix proteins<sup>275,429,430</sup>. Persistent or upregulated TGF- $\beta$  production in CAN, however, contributes to fibrogenesis, with an accumulation of extracellular matrix and inhibition of its degradation. In CAN, TGF- $\beta$  is expressed in the glomerular mesangium, Bowman's capsule, renal tubule and interstitium, the level of mRNA expression correlating well with interstitial fibrosis and progressing allograft nephropathy<sup>259,269,431-436</sup>. Although a direct upregulation of TGF- $\beta$  by Shh has not been demonstrated, members of the TGF- $\beta$  superfamily members including the bone morphogenetic proteins (BMPs)<sup>4,119,427</sup> and, in *Drosophila*, the morphogen *Decapentaplegic* (*Dpp*) are downstream targets of Hh signalling. The segment polarity gene *Wingless* (*wg*) pathway<sup>437</sup> is also downstream of Hh signalling<sup>437,438</sup> and interactions between the *wg* and TGF- $\beta$  signalling pathways have been described<sup>117,120</sup>. Furthermore, at sites of fibrosis in the lung, both TGF- $\beta$  and Shh expression were apparent in the epithelium<sup>149</sup> suggesting that the two pathways may communicate during fibrogenesis.

VEGF is an endothelial mitogen produced by a number of cell types including epithelial cells and macrophages<sup>439-441</sup>. In addition to its mitogenic and angiogenic properties, it also causes an increase in vascular permeability<sup>442</sup> and acts as a chemoattractant for monocytes<sup>443,444</sup>. VEGF is expressed constitutively in the normal kidney where mRNA and protein are co-localised to the podocytes and ductal

epithelial cells<sup>445-449</sup>. In chronically, but not acutely, rejecting human renal allografts VEGF expression has also been demonstrated in the macrophages of the tubulointerstitial cell infiltrate and in viable podocytes<sup>265,266,450</sup>. A direct association between Shh signalling and VEGF has been shown with recent reports suggesting that administration of Shh upregulates VEGF in myofibroblasts<sup>102</sup> and induces capillary morphogenesis in cultured endothelial cells<sup>65</sup>. Furthermore, in the zebrafish embryo, the injection of Shh mRNA induced VEGF expression in the somites<sup>163</sup>.

Thus, the initial aims of the work described in this chapter were 2-fold. The first aim was to evaluate the histology, cellular infiltration and growth factor expression in a population of normal human kidney samples and samples of CAN to establish whether the sample set used here demonstrated similar patterns of expression and histological phenotypes to those published previously. The second aim of this work was to evaluate the expression of the Shh signalling pathway components, Shh and Ptc in the same samples of both normal kidney and CAN kidney.

## 3.2 The hallmarks of CAN

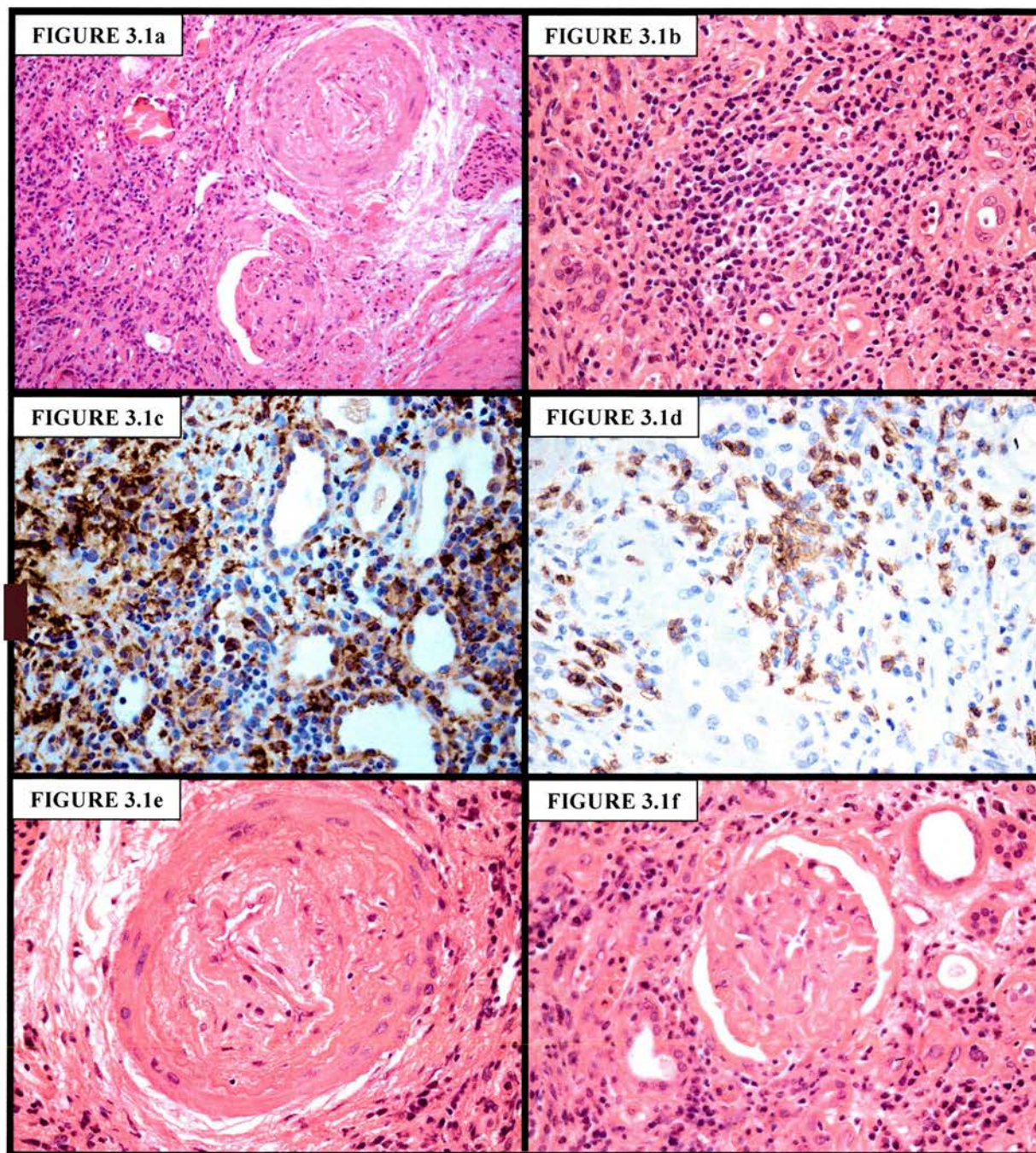
It has been shown previously that chronic renal allograft nephropathy (CAN) is characterised histologically by interstitial fibrosis, tubular atrophy, an inflammatory cell infiltrate consisting mainly of macrophages and T cells, glomerulopathy and vasculopathy<sup>226,227,246</sup>. This section of work aimed to establish whether, in a selected population of CAN specimens, the same histopathological features are evident.

CAN samples were obtained from 3 patients undergoing transplant nephrectomy for chronically failing allografts. Written and verbal consent to the use of the renal tissue for the purposes of these experiments was obtained from each patient. A further 10 FFPE CAN specimens were obtained from archival transplant nephrectomy material. As previously, ethical approval for these studies was obtained from the Lothian Research Ethics Committee.

Immunohistochemistry was performed on 3µm sections of FFPE tissue as described in Chapter 2. Haematoxylin-eosin staining was performed to allow the gross morphological features of CAN to be identified. Picrosirius red was used to visualise collagen present in the sections, as described previously<sup>422</sup> and as outlined in Chapter 2. Viewed with non-polarised light, the fibres appear red. Mouse monoclonal anti-human antibodies to CD68 (1:20 dilution) and CD3 (1:30 dilution) (Dako Ltd) were employed to detect macrophages and T cells respectively. For the immunohistochemical detection of CD68 and CD3, tissue sections were subjected to antigen retrieval using Vector antigen retrieval solution (Vector Laboratories, Peterborough, England) and avidin/biotin blockade (Avidin/Biotin blocking kit, Vector Laboratories). Non-specific binding was blocked using Dako serum-free protein block (Dako Ltd, Ely, UK). The sections were incubated for 1 hour at room temperature with the appropriate primary antibody. Following washing, the secondary antibody was applied for 1 hour. The secondary antibody for both primary antibodies was a biotinylated anti-mouse IgG antibody (Dako Ltd) used at 1:400 dilution. Positive signalling was identified using diaminobenzidine (Dako Ltd).

Representative results are shown in Figures 3.1 and 3.2. Figure 3.1 demonstrates some of the hallmarks of CAN (Figure 3.1a), notably an inflammatory cell infiltrate (Figure 3.1b), consisting of macrophages (Figure 3.1c) and T cells (Figure 3.1d), allograft vasculopathy (Figure 3.1e) and glomerulopathy (Figure 3.1f). Picrosirius red staining, demonstrating marked fibrosis in CAN, is shown in Figure 3.2a. A section of normal kidney, also stained with picrosirius red, is shown for comparison (Figure 3.2b).



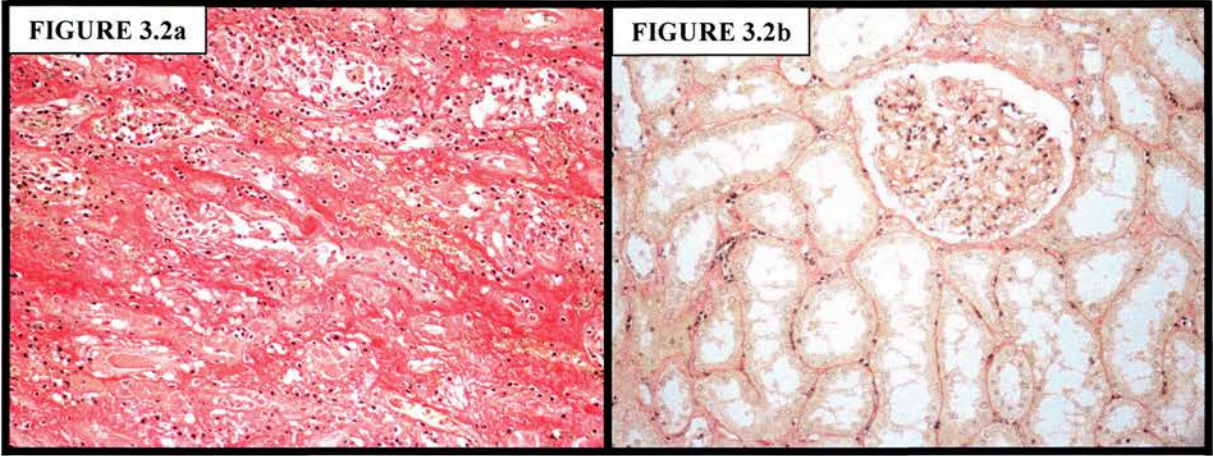


**FIGURE 3.1**

**CAN is characterised by an inflammatory cell infiltrate, tubular atrophy, allograft vasculopathy and glomerular sclerosis**

Immunohistochemistry was performed on 13 sections of CAN derived from allograft nephrectomy material. Haematoxylin-eosin (H&E) staining was performed to allow the gross morphological features of CAN (Figure 3.1a, Mag x 200) to be identified. H&E staining confirmed the presence of tubulointerstitial inflammatory cell infiltration (3.1b, Mag x 400), and anti-CD68 (Figure 3.1c, Mag x 400) and anti-CD3 (Figure 3.1d, Mag x 400) staining confirmed the presence of macrophages and T cells respectively in the inflammatory cell infiltrate. H&E staining was also used to demonstrate the presence of allograft vasculopathy (Figure 3.1e, Mag x 400) and glomerulopathy (Figure 3.1f, Mag x 400).





**FIGURE 3.2**

**CAN is characterised by fibrosis**

Fibrosis was evaluated using picosirius red staining on sections of CAN derived from allograft nephrectomy material. Collagen fibres appear red when stained with picosirius red and Figure 3.2a (Mag x 200) illustrates the marked collagen deposition seen in CAN. A section of normal kidney stained with the picosirius red is shown for comparison in Figure 3.2b (Mag x200)

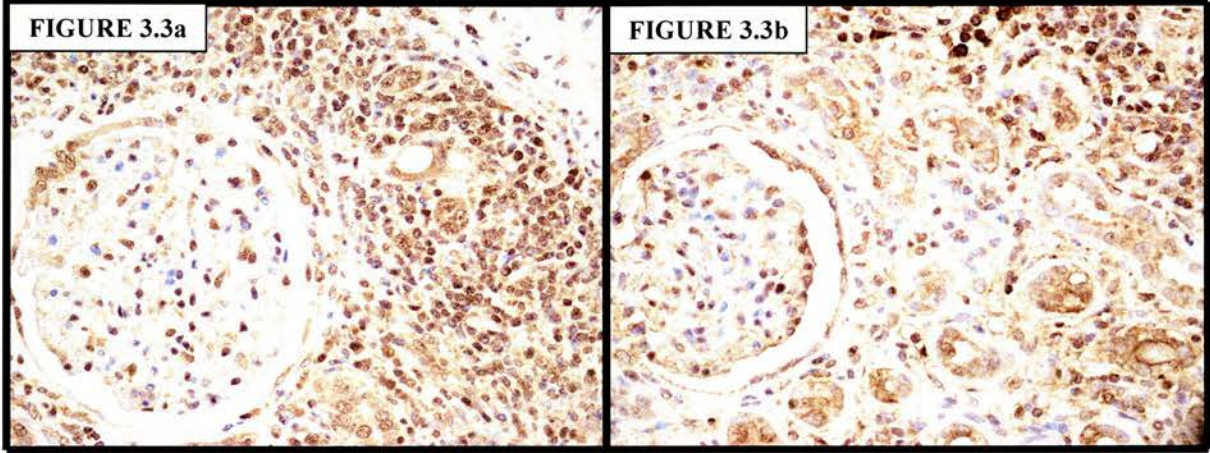
### 3.3 TGF- $\beta$ and VEGF are expressed in CAN

In addition to the histological hallmarks of CAN, a number of growth factors are also believed to be expressed and play a role in CAN. These factors include TGF- $\beta$ <sup>269,432,434-436</sup>, VEGF<sup>265,266,450</sup>, platelet-derived growth factors (PDGF)<sup>262</sup> and basic fibroblast growth factor (bFGF)<sup>451</sup>. Of these, TGF- $\beta$  and VEGF warrant further consideration given their possible relationship with, and regulation by, the Shh signalling pathway. Thus, in this section of work, the expression of TGF- $\beta$  and VEGF was evaluated in a series of CAN specimens.

As above, CAN samples were obtained from 3 patients undergoing transplant nephrectomy for chronically failing allografts. A further 10 FFPE CAN specimens were obtained from archival transplant nephrectomy material. The sections were evaluated immunohistochemically using a chicken polyclonal anti-human TGF- $\beta$ 1 (1:50 dilution) (R&D Systems, Abingdon, UK) and a goat polyclonal anti-human VEGF (1:40 dilution) (Santa Cruz Biotechnology) to detect TGF- $\beta$ 1 and VEGF respectively. Sections were incubated in the appropriate primary antibody overnight at 4°C. Following washing, the secondary antibody was applied for 1 hour. The secondary antibodies were a biotinylated anti-chicken IgG antibody (Vector) used at 1:300 and a biotinylated anti-goat IgG antibody (Dako Ltd) used at 1:400. Positive signalling was identified using diaminobenzidine (Dako Ltd).

In keeping with previous reports in the literature, TGF- $\beta$  was found to be expressed in the glomeruli and tubulointerstitium (Figure 3.3a) and VEGF was expressed in the tubulointerstitial cell infiltrate and in viable podocytes (Figure 3.3b) in CAN.

Thus, taken together with the results presented in Section 3.2, these results confirm that the population of CAN specimens utilised here exhibits similar features in terms of histopathology, cellular infiltration and TGF- $\beta$  and VEGF expression to those described in earlier studies<sup>259,265,266,431,432,434-436</sup>.



**FIGURE 3.3**

**TGF- $\beta$  and VEGF expression are evident in CAN**

Polyclonal chicken and goat antibodies were used to detect TGF- $\beta$  and VEGF respectively. TGF- $\beta$  (Figure 3.3a, Mag x 400) and VEGF (Figure 3.3b, Mag x 400) are both expressed in CAN in the glomeruli and tubulointerstitium in keeping with previous reports in the literature.

### 3.4 Shh is expressed in normal adult human kidney

Although Shh expression has been reported in the metanephric kidney<sup>2,4,138,139,428</sup>, there have been no previous reports of Shh expression in the normal adult human kidney. Therefore, the aim of these studies was to determine whether Shh and its receptor Ptc are expressed in normal adult human kidney.

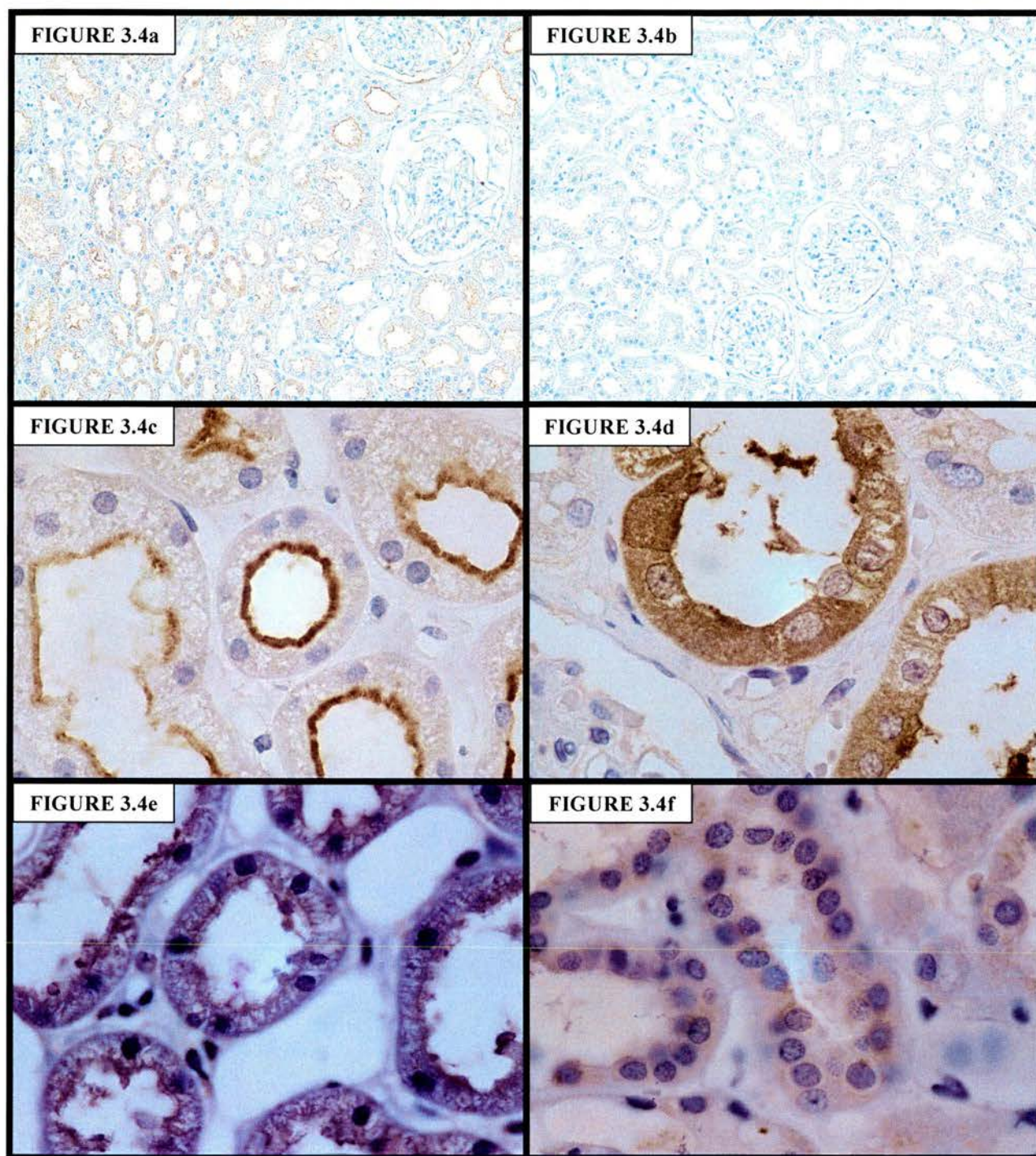
Normal human renal tissue was obtained from 3 cadaveric transplant donors whose kidneys were not placed for transplantation. Fresh tissue from these kidneys was fixed in formalin prior to paraffin embedding. A further 7 formalin-fixed, paraffin embedded (FFPE) archival samples from non-diseased parts of kidneys nephrectomised for renal tumours were also available for analysis. Ethical approval for these studies was obtained from the Lothian Research Ethics Committee.

Immunohistochemistry was performed on 3µm sections of FFPE tissue as described in Chapter 2. All tissue sections were subjected to antigen retrieval using Vector antigen retrieval solution (Vector Laboratories, Peterborough, England) and avidin/biotin blockade (Avidin/Biotin blocking kit, Vector Laboratories). Non-specific binding was blocked using Dako serum-free protein block (Dako Ltd, Ely, UK). The sections were incubated overnight at 4°C with antibodies to Shh and Ptc. These were goat polyclonal antibodies to the N-terminus of Shh and C-terminus of Ptc (Santa Cruz Biotechnology, Insight Biotechnology Ltd, Wembley, Middlesex, UK) and were used at 1:40 and 1:30 dilution respectively, as described previously<sup>149</sup>. To check antibody specificity, a further aliquot of each antibody was preincubated overnight with its specific blocking peptide. These blocking peptides are designed to bind to the epitope of the antibody responsible for binding to the protein of interest and, thus, when used in excess, should not allow binding of the antibody to the specific protein to occur. In accordance with the manufacturer's instructions (Santa Cruz Biotechnology), the antibodies were each pre-incubated with a five-fold excess (by weight) of blocking peptide and then applied to the sections in parallel with slides in which primary antibodies alone were applied. Tissue sections treated in this way served as negative controls. The secondary antibody for both primary antibodies

was a biotinylated anti-goat IgG antibody (Dako Ltd) used at 1:400. Positive signalling was identified using diaminobenzidine (Dako Ltd).

Figure 3.4 illustrates the results from these experiments. Shh (Figure 3.4a), but not Ptc (Figure 3.4b), expression was apparent in all 10 sections of normal adult human kidney. In all sections, there was focal staining for Shh in the brush border of the proximal convoluted tubules (PCT) (Figure 3.4c). In 6 of the 10 samples, this was in association with isolated diffuse cytoplasmic staining of the epithelium of the PCT (Figure 3.4d). All staining was significantly abrogated using the specific blocking peptide for Shh (Figures 3.4e and 3.4f).





**FIGURE 3.4**

**Shh, but not Ptc, is expressed in normal adult human kidney**

Immunohistochemistry was performed on 10 sections of normal adult human kidney using goat polyclonal antibodies to the N-terminus of Shh and the C-terminus of Ptc at dilutions of 1:40 and 1:30 respectively. Although all 10 sections demonstrated focal expression of Shh (Figure 3.4a, Mag x 200), Ptc was not expressed in any of the sections (Figure 3.4b, Mag x 200). In all 10 sections, focal expression of Shh on the brush border of the proximal convoluted tubule was apparent (Figure 3.4c, Mag x 1000). In 6 of the samples, this was in association with isolated diffuse cytoplasmic staining of the epithelium of the proximal convoluted tubule (Figure 3.4d, Mag x 1000). This brush border (Figure 3.4e, Mag x 1000) and cytoplasmic staining (Figure 3.4f, Mag x 1000) could be blocked using the specific Shh blocking peptide.

### **3.5 Ptc, but not Shh, is expressed in CAN**

Given that upregulated Shh expression has been demonstrated in human idiopathic pulmonary fibrosis and murine models of pulmonary fibrotic disease<sup>149</sup>, the following experiments were performed to determine whether Shh and Ptc expression were evident in CAN.

#### **3.5.1 Immunohistochemistry**

Using the same CAN specimens as those used in Sections 3.3, immunohistochemistry was performed to determine the expression of Shh and its receptor, Ptc. Goat polyclonal antibodies to the N-terminus of Shh and C-terminus of Ptc (Santa Cruz Biotechnology) were used at 1:40 and 1:30 respectively and specificity was confirmed using the appropriate specific blocking antibodies.

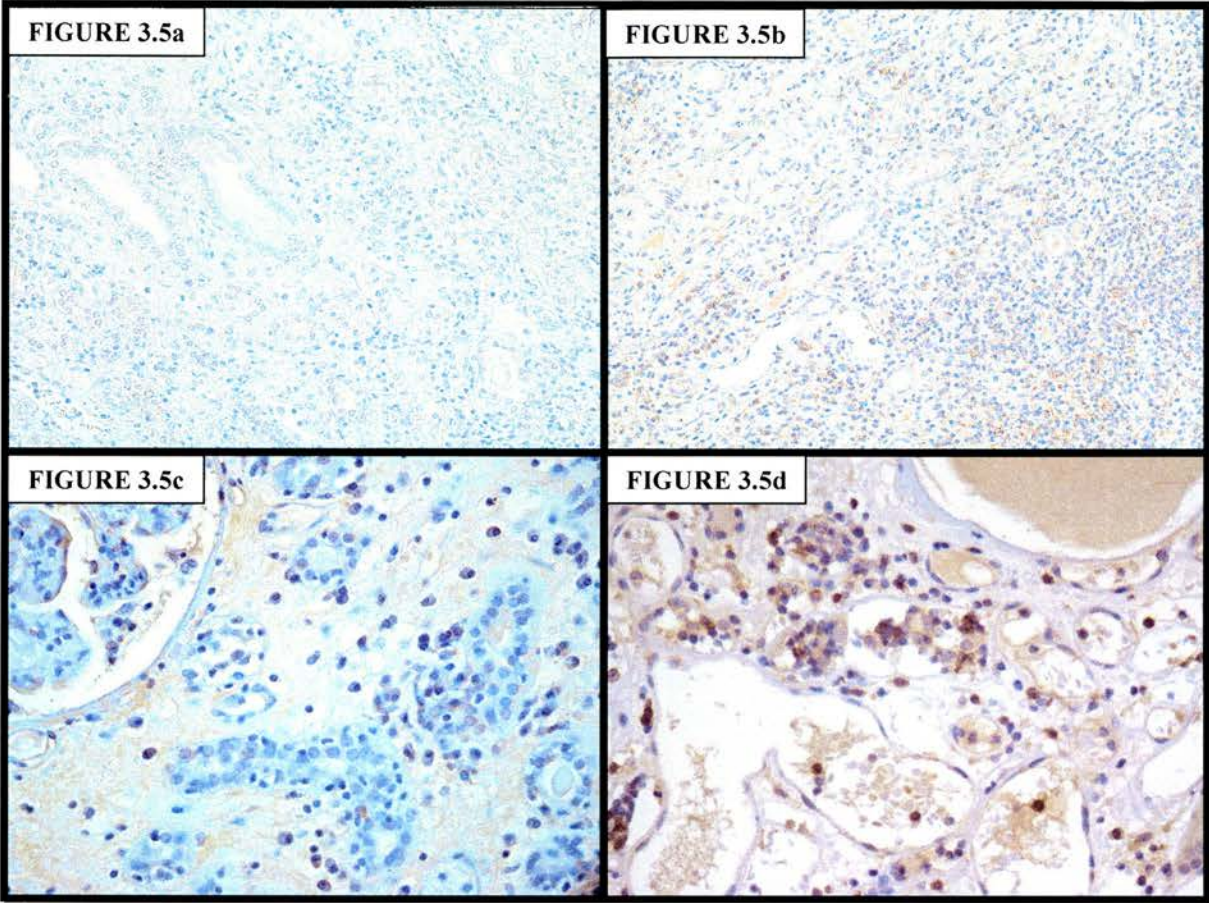
The results from the immunohistochemical studies are shown in Figure 3.5. There was a complete absence of Shh staining across all specimens examined (Figure 3.5a and c). In contrast, Ptc was detected on infiltrating inflammatory cells (Figure 3.5b and d) in all CAN sections.

#### **3.5.2 Real-time PCR**

In addition to the immunolocalisation studies described above, RNA was extracted using Trizol extraction from the normal kidneys not used for transplantation (n=3) and from the kidneys nephrectomised for chronically deteriorating graft function (n=3). RNA was reverse transcribed to cDNA. Real-time PCR was then performed on each sample in duplicate using 18S RNA as an internal control to compare the relative expression of *Shh* and *Ptc* in normal and CAN kidney. Values are expressed as a ratio of the concentration of *Shh* and *Ptc* mRNA in CAN relative to normal kidney, where normal kidney is given a value of 1.

The results are shown in Figure 3.6. While *Ptc* expression is upregulated in CAN (1.6-fold), *Shh* expression is lower (5-fold) than that seen in normal kidney. Thus, these results corroborate the protein results obtained using the immunohistochemistry outlined above in Section 3.5.1.

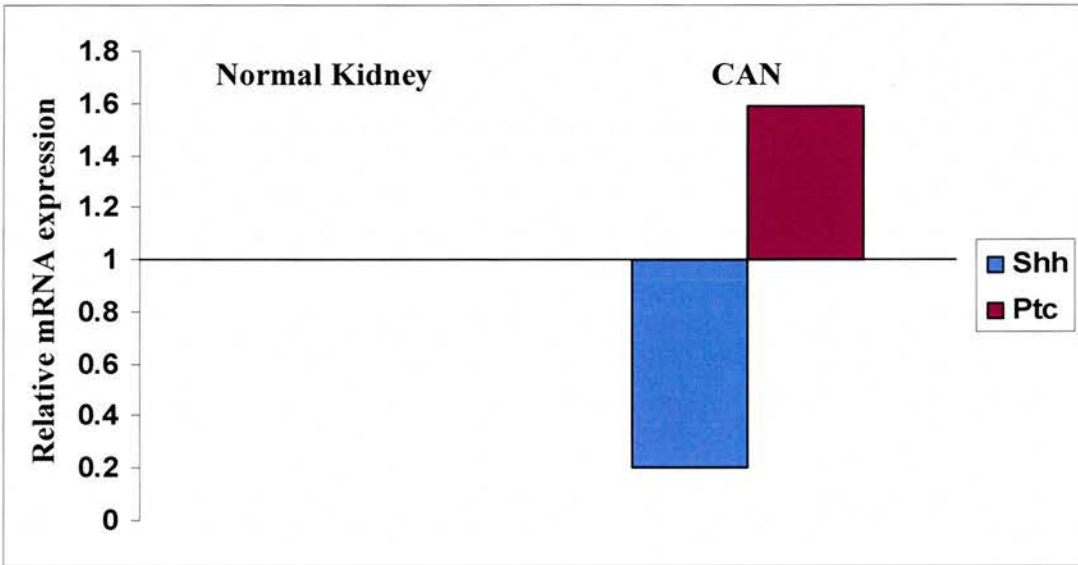




**FIGURE 3.5**

**Ptc, but not Shh is expressed in CAN**

Immunohistochemistry was performed on 13 sections of CAN derived from allograft nephrectomy material using polyclonal antibodies to Shh and Ptc as previously. There was a complete absence of Shh staining across all specimens examined (Figure 3.5a, Mag x 200 and Figure 3.5c, Mag x 400). In contrast, Ptc was detected on the infiltrating inflammatory cells (Figure 3.5b, Mag x 200 and Figure3.5d, Mag x 400) in all CAN sections.



**FIGURE 3.6**

***Shh* and *Ptc* mRNA expression in normal kidney and CAN**

Real-time PCR was performed to determine the relative expression of *Shh* and *Ptc* mRNA in normal kidneys (n=3) and in kidneys nephrectomised for chronically deteriorating graft function (n=3). RNA was reverse transcribed to cDNA. Real-time PCR was then performed on each sample in duplicate using 18s as an internal control to compare the relative expression of *Shh* and *Ptc* in the normal and CAN kidneys. Values are expressed as a ratio of the concentration of *Shh* and *Ptc* mRNA in CAN relative to normal kidney, where normal kidney is given a value of 1. The results presented are representative of 3 independent experiments.

### **3.6 Shh and Ptc expression are not apparent in renal biopsy specimens**

That Shh is expressed in normal kidney but that this expression is lost in CAN is interesting and contrasts with the upregulated Shh expression seen in human fibrotic lung disease and murine models of fibrotic lung disease<sup>149</sup>. The work of Stewart et al exploring the expression of Shh and Ptc in fibrotic pulmonary disease demonstrated that Shh is upregulated at sites of active epithelial inflammation overlying areas of fibrosis. Thus, it may be that Shh upregulation occurs in response to injury and, indeed, it has been postulated that Shh may play a role in either repair and/or remodelling processes<sup>149,161</sup>. Other reports in the literature would lend support for such a role. For example, the application of recombinant Shh to endothelial cells has been shown to induce capillary morphogenesis<sup>65</sup> and, in the hindlimb ischaemia model, Shh was shown to induce angiogenesis, improving the blood flow to the ischaemic limb<sup>102,103</sup>. Furthermore, Hh signalling may be involved in fracture repair as evidenced by studies reporting the activation of Hh signalling under these conditions<sup>152,153</sup>.

In light of these reports implicating an early role for Hh signalling in inflammatory process and/or repair, it is perhaps not then surprising that, in CAN, characterised by end-stage epithelial damage and tubular loss, Shh expression is lost. If indeed Shh signalling is more relevant in the acute stages of renal disease, investigating the effects of earlier insults to the allograft may be more relevant and informative. In this regard, 2 other renal pathologies, acute rejection (AR) and acute tubular necrosis (ATN) were chosen for further investigation.

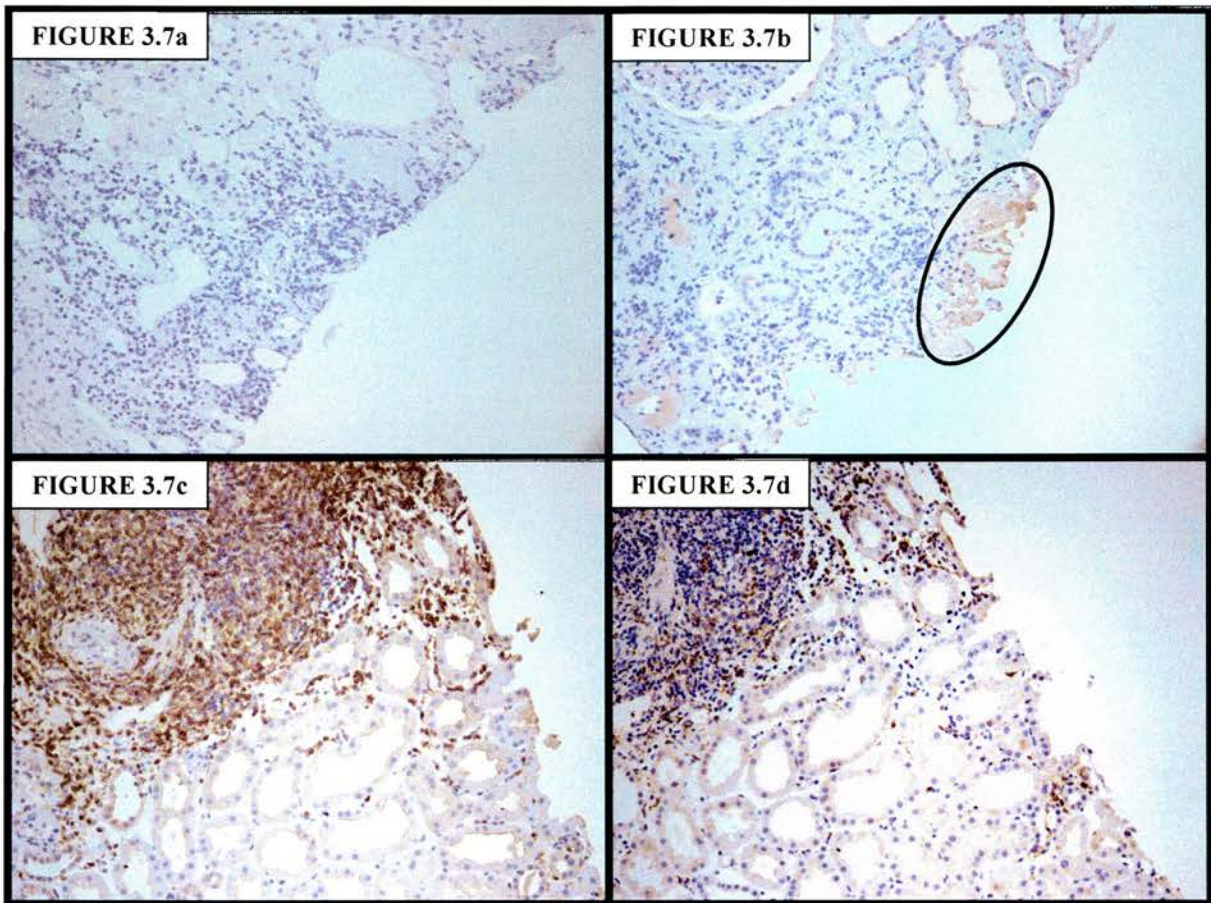
During the early course following allograft implantation, a deterioration in graft function most commonly reflects either AR or ATN. Both pathologies effect changes in the tubule. AR is characterised by tubulitis, i.e. mononuclear cell infiltration between or beneath tubular cells<sup>227</sup> and arteritis. ATN is characterised by loss of the brush border, interstitial oedema and an interstitial inflammatory cell infiltrate. Thus,

both pathologies represent instances in which, if Shh is involved in tubular inflammation or repair, altered expression of Shh may be exhibited.

Therefore, the aim of the following experiments was to evaluate Shh and Ptc expression in both AR and ATN. Archival material in the form of 20 diagnostic biopsies, taken 7 days post-transplantation, was available for immunohistochemical analysis. Ten biopsies had been reported as ATN and a further 10 as AR. Of the latter 4, 2, 3 and 1 were graded as Grades 1a, 1b, 2a and 2b acute cellular rejection respectively. As in the earlier sections of this chapter, the biopsies were investigated for Shh and Ptc expression using goat polyclonal antibodies (Santa Cruz) to Shh and Ptc at 1:40 and 1:30 respectively. In each run of immunohistochemistry, a sample of normal kidney was run in parallel as a positive control. As previously, antibodies pre-incubated with the respective blocking antibody acted as the negative controls.

Representative results are shown in Figure 3.7. Neither Shh (Figure 3.7a) nor Ptc were apparently expressed in any of the biopsy sections (Figure 3.7b). There was, however, a marked edge effect around many of the sections examined. This could not be eliminated despite further optimisation of the immunohistochemistry protocol. In contrast, staining of the biopsy samples using monoclonal anti-CD3 (Figure 3.7c) or anti-68 (Figure 3.7d) antibodies in the same specimens revealed good quality staining in the absence of non-specific staining or artefacts. Therefore, from these results, it is not clear whether the absence of Shh expression reflects a true lack of expression or an artefact resulting from either tissue fixation or inadequate immunohistochemical techniques.





**FIGURE 3.7**

**Shh and Ptc expression are not apparent in renal biopsy specimens**

Immunohistochemistry was employed to determine Shh and Ptc expression in 10 acute rejection and 10 acute tubular necrosis biopsy specimens. Neither Shh (Figure 3.7a, Mag x 200) nor Ptc (Figure 3.7b, Mag x 200) were apparently expressed in any of the biopsy specimens and, indeed, a marked edge effect was noted around some of the biopsies (Figure 3.7b, circled). In contrast, staining of the biopsy samples using monoclonal anti-CD3 (Figure 3.7c, Mag x 200) or anti-68 (Figure 3.7d, Mag x 200) antibodies in the same specimens revealed good quality staining in the absence of non-specific staining or artefacts.

### 3.7 Discussion

The work presented in the first part of this chapter has used immunohistochemistry on samples of adult human kidney, removed after transplant failure due to CAN in order to investigate the histopathology, cellular infiltration and growth factor expression in CAN. Indeed, this work has confirmed, in this sample, the presence of the hallmarks of CAN including interstitial fibrosis, vasculopathy, glomerulopathy and a mononuclear cell infiltration. In addition, concentrating on the cell types and growth factors specifically relevant to this thesis, this work has confirmed that both macrophages and T cells are present in the CAN kidney and that TGF- $\beta$  and VEGF are expressed. These results show that, not only do the samples used in this study exhibit the histopathological hallmarks of CAN, but, also, that similar patterns of growth factor expression to those reported previously can be demonstrated.

In the second part of the chapter, immunohistochemistry has been used further in association with real-time PCR, to investigate the expression of the Shh signalling pathway components Shh and Ptc in samples of both normal adult human kidney and CAN. The findings from this investigation are discussed below:

#### 3.7.1 Shh is expressed in normal adult human kidney but not CAN

Although Shh expression has been described previously in the embryonic kidney, the results presented in this chapter are the first to indicate that Shh is expressed in the normal adult human kidney. In all sections of normal kidney tested, Shh expression was detected on the brush border of the proximal convoluted tubule (PCT). In 6 of the 10 samples, this was in association with isolated diffuse cytoplasmic staining of the epithelium of the PCT. All staining was significantly abrogated using the specific blocking peptide for Shh. In contrast, Ptc protein expression was not detected in normal kidney. Conversely, although Shh expression is lost in CAN, Ptc is expressed on cells within the inflammatory cell infiltrate. Although the precise inflammatory cell type responsible for Ptc expression is not clear, both T cells<sup>20,21</sup> and macrophages<sup>149</sup> are known to express Ptc and both cell types are present within

the inflammatory cell infiltrate seen in CAN. The differential expression of Shh and Ptc in the CAN versus normal kidney is further confirmed by the results of the real-time PCR experiments.

The reasons for these patterns of expression are not immediately apparent. Given that Shh expression upregulation has been reported in epithelium overlying areas of fibrosis in murine models of fibrotic lung disease and in idiopathic pulmonary disease in humans<sup>149</sup>, it is perhaps surprising that Shh expression is lost in CAN, a disease characterised by extensive fibrosis. However, that Shh expression is localised to the proximal tubular cells in normal kidney may help, at least in part, to explain this absence of expression in CAN since tubular atrophy is one of the earliest changes observed in diagnosing CAN and is a feature of all but the mildest forms of CAN<sup>227</sup>. Thus, although occasional tubules did appear macroscopically intact in the CAN kidneys and these were negative for Shh expression, it is extremely likely that the loss of tubular expression may reflect an absence of viable or healthy tubules. Indeed, even tubules with apparently normal appearance by light microscopy can be markedly dysfunctional<sup>452</sup>.

Although Shh expression has been reported previously in the developing kidney<sup>2,4,139</sup>, the pattern of expression in the adult is not in keeping with the pattern seen in the embryo. In the embryonic metanephric kidney, the uroepithelium of the collecting ducts and the ureter express Shh<sup>2,4,138,139</sup>. In contrast, neither proximal tubular cytoplasmic nor brush border Shh expression have been previously reported in the developing kidney. Thus, the tubular and brush border expression reported here in normal kidney represent particularly interesting findings, although one can only speculate on the reason for this pattern of expression. Although Shh expression has not been detected in the adult human kidney, another of the Hedgehog homologues, Ihh, has been reportedly expressed in the adult kidney<sup>2,146</sup>. Notably, studies by Valentini et al<sup>146</sup> reported on *Ihh* mRNA expression in proximal convoluted tubule of the murine kidney. Thus, there is the possibility that the antibody directed against the N-terminus of Shh may also be capable of detecting Ihh. Indeed, murine Ihh has been demonstrated to show 89% identity and 96%

similarity to murine Shh over the N-terminal region. In addition, all Hh proteins have been shown to bind Ptc with equal affinity<sup>453</sup> and proteolysis of Ihh gives rise to a 19kDa active peptide in a similar proteolytic cleavage process to that occurring for Shh<sup>146</sup>. Therefore, given these similarities, one possibility is that the Shh antibody is recognising Ihh rather than Shh. However, that the antibody is reported by the manufacturer (Santa Cruz Biotechnology) to be non-cross-reactive with Ihh or Desert hedgehog (Dhh) would not lend support to this idea. A further possibility also exists; that the brush border binding of antibody and indeed PCT binding represent artefacts of immunohistochemical staining. However, 2 lines of evidence would lend support for this staining being neither artefactual nor non-specific. Firstly, the staining was significantly abrogated using the specific Shh blocking peptide, a peptide specifically designed to bind to the epitope of the antibody responsible for binding to the protein of interest. Secondly, the immunohistochemistry data is corroborated by the real-time PCR results. These demonstrated a 5-fold difference in *Shh* mRNA expression between normal and CAN kidney which is in keeping with the respective presence and absence of Shh protein in the normal kidney and CAN sections.

### **3.7.2 Possible roles for Shh in normal kidney**

Taken together, the findings prompt speculation regarding a possible role for Shh in the epithelium of the normal adult kidney. Shh typically effects its action on target cells through binding to its receptor Ptc<sup>40,100</sup>. It is therefore of particular interest that Ptc protein expression was not apparent in any of the sections of normal kidney. That this is the case did not appear to represent inadequate optimisation of the Ptc antibody for this purpose since Ptc expression was clearly detected on the infiltrating inflammatory cells in the sections of CAN, which, for practical purposes, were run in parallel experiments with the immunohistochemistry on the normal kidney sections.

During development of the metanephric kidney, upregulation of Ptc in the mesenchyme surrounding the distal collecting ducts and the ureter suggests that Shh functions via Ptc in a paracrine manner. Indeed, *in vivo* and *in vitro* analyses demonstrate that Shh signalling through Ptc promotes mesenchymal cell



proliferation, regulates the timing of differentiation of smooth muscle progenitor cells, and sets the pattern of mesenchymal differentiation<sup>138</sup>. However, the absence of Ptc protein expression in the adult kidney would suggest that either protein expression is absent or that its expression is so low as to evade detection by the Ptc antibody. That *Ptc* mRNA could be detected by real-time PCR lends support to the latter although it is possible that, under certain circumstances, *Ptc* mRNA expression is upregulated, and translated into upregulated protein expression, allowing Shh signalling through the Shh-Ptc axis to occur. Alternatively, Shh may signal via a different receptor. For example, Shh can bind to the less well characterised Ptc receptor, Ptc2<sup>128,130</sup> or the membrane glycoprotein Hedgehog interacting protein (Hip). Hip, like Ptc, is a transcriptional target of Hh<sup>124</sup> although it remains to be determined whether either Ptc2 or Hip are expressed in the kidney. Alternatively, it may be that Shh, if functional in the kidney, acts independently of the Ptc-Smo-Gli axis altogether. Such pathway independence has been demonstrated recently in investigations into the effect of Shh on cell adhesion and migration of neural crest cells<sup>121</sup>. Testaz et al<sup>121</sup> posited that the effect of Shh on neural crest cells may be mediated, at least in part, through interactions with members of the integrin family since Shh failed to exert its effect in  $\beta$ 1-integrin-deficient mice. Indeed, it is interesting to note that interactions between Shh and the extracellular matrix protein, vitronectin, have also been described<sup>454</sup>.

A further possibility arises out of a recent discovery suggesting that Shh can bind to megalin<sup>126</sup>. Megalin, originally recognised as the Heymann nephritis autoantigen, gp330<sup>455,456</sup>, and then, subsequently, as a member of the LDL receptor family<sup>293,457</sup>, is one of the most abundant proteins in the renal proximal tubule (PCT)<sup>135,458</sup>. Indeed, expression of megalin on the apical surface of proximal tubular cells has been demonstrated in preliminary experiments in 2 samples of normal kidney (See Appendix 1), a finding in keeping with previous reports<sup>459</sup>.

Megalin functions as a receptor for numerous ligands<sup>460</sup>. These include apolipoprotein E-enriched lipoprotein particles, complexes composed of urokinase and tissue-type plasminogen activator in complex with type-1 inhibitor (PAI-1)<sup>461</sup>,

receptor-associated protein (RAP)<sup>462</sup>, vitamin D<sup>463</sup> and calcium, as well as vitamin B12<sup>464,465</sup>, albumin and polypeptide hormones. One of the key functions of megalin is to mediate endocytosis and transepithelial transport of ligands in the kidney, targeting them for either lysosomal degradation or transcytosis<sup>459,466</sup>.

In this chapter, it has been shown that Shh, like megalin, is expressed on the apical surface of the PCT, this close proximity suggesting the possibility of a Shh-megalin interaction. It is also notable that both Shh and megalin are expressed in the lung<sup>135,149</sup>. In the adult lung, megalin expression has been localised to the type II pneumocytes<sup>135</sup> and, indeed, these same cells exhibited upregulated Shh expression in fibrotic pulmonary disease in the human and a mouse model<sup>149</sup>. It remains to be seen whether this interesting association in the lung and kidney represents an underlying functional mechanism or merely an irrelevant coincidence. If, indeed, the Shh and megalin interaction is important in the kidney, it is tempting to speculate as to its possible role. One possibility is that Shh protein is present in the glomerular filtrate and is endocytosed by megalin binding it to the apical surface of the PCT. If this is the case, it is not clear why this would occur. Urine is not known to contain Shh, although this could be readily evaluated, at least in mice, using the commercially available murine Shh ELISA. It is also conceivable that the Shh antibody is detecting Shh protein bound to megalin on the surface of the cells and either free Shh, or Shh in association with megalin in the cytoplasm of the PCT. Although an interesting possibility, this is unlikely to represent the whole picture since this mechanism would not explain the relatively high levels of *Shh* mRNA expression in normal kidney relative to CAN kidney. Thus, it seems possible that at least some contribution to the epithelial Shh arises from *de novo* synthesis of *Shh* mRNA.

Another issue of interest here relates to the possible implications of an interaction between Shh and megalin. It has been postulated that megalin may modulate Ptc and Smo trafficking<sup>127</sup>. Indeed a number of studies report low levels of Ptc on the surfaces of cells and that the majority of Ptc protein is present in the intracellular endosomal compartments<sup>57,58</sup>. It is possible that low levels of surface Ptc and

increased endosomal accumulation may result from megalin-mediated endocytosis, a scenario analogous to the roles of other members of the LDL receptor family<sup>467</sup>. It is of further interest then that studies investigating the interaction of Ptc and Smo in endosomes used a renal epithelial cell line for this investigation<sup>52</sup>. Indeed, Incardona et al<sup>52</sup> demonstrated Ptc-Smo complex internalisation following binding of Shh ligand in this model. The failure of Ptc protein to be detected in the kidney by immunohistochemistry, despite the presence of *Ptc* mRNA would be in keeping either with low levels of expression or masking of the Ptc, by megalin, Smo or Shh, to the detection antibody.

Although megalin appears to play a role in mediating endocytosis, it is not entirely clear whether megalin binding is capable of directly inducing signal transduction pathways. However, other members of the LDL receptor family do appear to mediate signalling<sup>467</sup>. Interestingly, some mediate signalling through the phosphoinositide 3-kinase pathway<sup>468</sup>, a pathway also previously shown to be activated by Shh's interaction with Ptc in endothelial cells<sup>65</sup>. Thus, these interesting reports point to a possible role for Shh's interaction with megalin in the kidney. A final point of interest relates to the recent findings of Gekle et al<sup>469</sup> illustrating that enhanced levels of TGF- $\beta$ 1 lead to reduced megalin-cubilin-mediated endocytosis<sup>469</sup>. If Shh does bind to megalin in the epithelium of the PCT, it is possible that further effects stemming from this interaction may also be inhibited by TGF- $\beta$ . Indeed, given the upregulated TGF- $\beta$  in association with the extensive fibrosis seen in CAN, it is quite possible that this TGF- $\beta$  upregulation could have profound effects on Shh expression and possible Shh-megalín interactions. Interestingly, findings from preliminary experiments in which megalín protein expression was evaluated in CAN, suggested that megalín expression is lost in CAN (See Appendix 2). Whether this observation reflects the loss of the brush border and proximal convoluted tubule integrity seen early in CAN or, instead, parallels the loss of Shh as a ligand for megalín, is not known.

One can only speculate further as to the function of Shh in renal epithelium. Perhaps it has a role in maintaining cell viability, acting as a survival factor. Several reports

in the literature would lend support to such a role<sup>425,470-472</sup>. Indeed, Shh has been shown to induce the survival factor bcl-2<sup>425,471</sup> in keratinocytes and lymphocytes<sup>21</sup>. Both bcl-2 and bcl-xl are expressed in proximal tubular epithelial cells and, interestingly, recent reports have suggested that the balance of survival to proapoptotic factors following cadaveric renal transplantation may be critical to determining the early outcome of the graft<sup>473-475</sup>. For example, upregulated bcl-2 expression has been demonstrated in the tubular cells of renal transplant recipients without post-ischaemic renal failure following cadaveric renal transplantation compared to those with acute renal failure<sup>473</sup>. In contrast, acute allograft rejection has been associated with a shift in the ratio of bcl-2 to bax (a pro-apoptotic) factor in tubular epithelium and increased expression of p53 in tubular nuclei<sup>474</sup>. Furthermore, in cadaveric renal allografts, bax expression is more prevalent than in living donors, a finding which may contribute to the poorer graft survival seen in cadaveric compared to living donor kidneys<sup>475</sup>. If Shh is involved in the upregulation of bcl-2 in renal tubular epithelium, it is conceivable that it may help to maintain cell viability in these cells, possibly influencing the early outcome of renal damage. Such an immediate early function for Shh would perhaps help to further explain the loss of Shh expression in CAN. It is interesting to speculate further on the significance of this. For example, given the epithelial expression of Shh and its possible action on immune cells, perhaps Shh is functioning as an endogenous danger signal in keeping with the danger theory proposed by Matzinger in the 1990s<sup>476,477</sup>. Proposed candidates for this role already include the heat shock proteins (HSPs). Increased expression of the heat shock proteins and molecular chaperones is a ubiquitous feature of cells exposed to acute but typically transient, stress conditions. In a number of organisms, induced expression of HSPs can be a marker for the adaptation to cyclical environmental changes<sup>478</sup>. In contrast, constitutively high levels of inducible HSPs such as HSP70 are not commonly seen in mammals. However, in response to injuries such as ischaemia and/or reperfusion heat shock protein expression is induced<sup>479</sup>. Furthermore, the induction of heat shock proteins has been shown to protect against ischaemia/reperfusion injury<sup>480-483</sup>, at least some of this effect attributable to the induction of bcl-2 as well as that of HSP 70 and 90 and heme-oxygenase 1<sup>480,484</sup>. Whether Shh signalling has the potential to be involved in

cell viability and/or preconditioning responses in the kidney is not clear. Interestingly in this regard, several groups have suggested that the antiapoptotic role of certain types of preconditioning may be, in part, mediated by AKT signalling leading to the prevention of caspase activation<sup>485,486</sup>. AKT has been shown to promote cellular survival *in vivo*<sup>487</sup> and it is notable that recent work by Kanda et al<sup>65</sup> has suggested that, in endothelial cells, Shh induces AKT signalling. Thus, it can be seen that tissue-derived Shh has the potential to function as a survival factor through the activation of a number of different downstream pathways including the bcl family of proteins and AKT signalling. In light of some of the similarities Shh may share with the heat shock proteins (ie upregulated at sites of damage, capable of activating cells of the immune system and survival effector function), further investigation along such lines would provide interesting avenues for further enquiry.

Other feasible roles for Shh in the kidney include a role in the maintenance of a healthy differentiated phenotype in tubular cells. A similar such role for Shh signalling has been proposed in the gut<sup>67</sup> and it is conceivable that it may assume a similar role in the kidney. Renal injury results in morphological and functional changes in epithelial cells<sup>226,488</sup>. Tubular epithelial cells are particularly susceptible and appear to dedifferentiate during renal ischaemia. It would be interesting to determine whether such dedifferentiation is associated with a loss of tubular Shh expression. It is of note that members of the bone morphogenetic proteins (BMPs), recognised downstream targets of Hh signalling, are expressed during renal development<sup>489</sup> and in the adult kidney<sup>490</sup> and several lines of evidence suggest that, at least some of the BMPs, most notably BMP7, may be important in stimulating and maintaining a healthy differentiated epithelial cell phenotype in the kidney. Firstly, severe renal dysorganogenesis occurs in the BMP7-deficient mouse with an absence of nephron formation<sup>491</sup>. Secondly, recombinant BMP7 protein is capable of inducing epithelial differentiation with subsequent nephron formation in metanephric organ culture<sup>492</sup> and in metanephric mesenchymal cell cultures<sup>490</sup>. Furthermore, BMP7 mRNA was shown to be downregulated in ischaemic kidneys compared to normal kidneys<sup>490</sup> and the administration of recombinant BMP7 prevents the tubular interstitial nephritis caused by obstructive uropathy<sup>493</sup>, abrogates glomerular sclerosis



in diabetic nephropathy<sup>494</sup>, blunts renal fibrosis<sup>493</sup> and leads to the repair of severely damaged renal tubular epithelial cells in a mouse model of chronic renal injury<sup>273</sup>. The mechanism of BMP7's action appears to be preservation of epithelial cell phenotype, inhibition of epithelial-mesenchymal transdifferentiation and inhibition of injury-induced apoptosis<sup>494</sup>.

Aside from possible maintenance of cell viability and/or maintenance of the healthy epithelial phenotype, other roles for Shh may be relevant. For example, Shh may have key roles in both embryonic<sup>163</sup> and postnatal angiogenesis<sup>65,102,103</sup>. Indeed, endothelial cells appear to be able to respond to Shh signalling as evidenced by work in which Shh induced capillary morphogenesis in both murine brain capillary endothelial cells and human umbilical endothelial cells<sup>150</sup>. Thus, it is possible that Shh may play some role in maintaining the microvasculature of the kidney.

Although it is interesting to speculate on possible roles for Shh in the normal kidney, a further possibility has yet to be considered – namely that the kidneys used in this study may not represent the expression seen in normal kidney. Indeed, none of the samples were truly “normal”. Three of the samples were derived from kidneys not used for transplantation and 7 from the normal parts of kidneys nephrectomised for tumours. Of those kidneys not used for transplantation, 2 kidneys were from donors with significant vascular disease. All 3 had further been subjected to the trauma associated with death of the cadaveric organ donor, an invariably lengthy multi-organ retrieval process, perfusion with preservation fluids and cold and warm ischaemia. Given that Shh may be upregulated early in inflammation and/or injury, it may be that Shh is released from renal epithelium in response to these insults. Indeed, the proposed danger signals, the heat shock proteins are induced very early in response to damage both *in vivo* and *in vitro*<sup>495-498</sup>. Thus, the normal kidneys from these unused kidneys may well reflect a somewhat altered expression of Shh and Ptc. Furthermore, the other 7 kidney specimens had been derived from kidneys containing tumours and whether these may be considered to be representative of truly normal renal tissue is also not clear.

### 3.7.3 Summary

The work presented in this chapter has used immunohistochemistry to demonstrate the established features of CAN in a sample population of kidneys nephrectomised for CAN. The work has further demonstrated that, in keeping with other reports in the literature, the growth factors TGF- $\beta$  and VEGF are expressed in CAN. In the latter part of the chapter, immunohistochemistry has further been employed to evaluate the expression of components of the Shh signalling pathway in normal and CAN kidney. The results demonstrate that Shh, but not Ptc, is expressed in normal adult human kidney. The results also indicate that, contrary to the enhanced expression of Shh seen in other models of fibrosis and human pulmonary fibrotic disease<sup>149</sup>, Shh is not expressed in CAN. Conversely, Ptc expression is evident in CAN, with expression being limited to cells of the inflammatory cell infiltrate. Possible reasons for this pattern of expression are discussed, as are possible roles for Shh in the normal adult kidney.



## **Chapter 4**

**The effect of immunosuppressive agents and cell damage on Sonic hedgehog expression in renal epithelial cell cultures**

## 4.1 Introduction

The results from Chapter 3 have raised a number of interesting questions with regards the possible role for Shh signalling in the adult kidney. Expression at the mRNA or protein level clearly does not provide sufficient evidence for an active role for Shh signalling in the kidney but it does tempt speculation regarding the possible roles Shh could play in the normal kidney and possible reasons for its loss in the kidney affected by CAN. Indeed, for Shh expression to be absent in the transplanted kidney affected by CAN could reflect the result of one or many different disease processes or idiopathic interventions that an allograft is subjected to during its lifespan. All transplanted kidneys used in this work were originally from cadaveric donors and had been subjected to treatment with immunosuppressive agents; they had undergone one or more episodes of acute allograft rejection and had progressed to chronic allograft nephropathy. Furthermore, by the time of transplant nephrectomy, all immunosuppressive agents had been discontinued with the invariable result that acute rejection changes superimpose on the underlying nephropathy. Thus, it can be seen that the Shh expression seen in normal kidney may well have been lost at any stage during the lifespan of the allograft. That the kidney affected by CAN has been subjected to a large range of potential insults, all of which may have led to the loss of Shh expression in the setting of allograft nephropathy, opens up several lines of enquiry. In Chapter 3, an attempt was made to investigate the expression of Shh in sections of kidney affected by ATN or ACR. Whether the apparent absence of Shh expression demonstrated in these immunohistochemistry experiments truly reflects the failure of Shh to be expressed in these conditions or whether it reflects an artefact of the immunohistochemistry in these biopsy samples remains to be established. If the apparent absence of expression seen in ATN and ACR accurately represents the Shh expression in these conditions *in vivo*, these results would suggest that Shh expression is lost early in the allograft's life, prior to, or as a result of, injury sustained as a result of ATN or ACR. For this reason, 2 avenues were chosen for further study in this chapter, both of which represent possible insults sustained by the transplanted kidney early in its lifespan. Firstly, and as outlined in the first part of this chapter, the effect of immunosuppressive agents on

renal epithelial Shh expression has been evaluated since the immunosuppressive agents are employed very early in the course of the allograft's life. In the second part of the chapter the effect of damaging agents on renal Shh expression has been explored. This is of particular relevance since, not only has the expression of Hh signalling components been associated previously with epithelial damage, inflammation<sup>149</sup> and repair<sup>152</sup> processes, but also epithelial damage is invariably seen in transplant nephropathy.

## **4.2 The effect of the immunosuppressive agents ciclosporin and dexamethasone on Shh expression in ACHN cells**

Of the factors that might influence Shh expression, the effect of immunosuppressive agents presented an avenue of inquiry that could be readily explored. All of the transplant nephrectomy samples used in the immunohistochemistry experiments described in Chapter 3 had been subjected to an immunosuppressive treatment consisting of a ciclosporin and steroid-based regimen. Thus, the aim of this section of work was to determine whether ciclosporin and the steroid, dexamethasone, could influence the Shh expression of human renal epithelium. For these experiments, the human renal adenocarcinoma (ACHN) (European Collection of Cell Cultures, ECACC No: 88100508) cell line was used. This cell line has been used previously<sup>499</sup> as an *in vitro* model of renal tubular cells. The management of this cell line, together with the medium used for cell cultures, is described further in Chapter 2.

### **4.2.1 Ciclosporin upregulates Shh expression in ACHN renal epithelial cells**

ACHN cells were seeded at a density of 50,000 cells per well in 6 well plates. After 2 days, when the cells were 75% confluent, the cells were washed with fresh medium (complete DMEM) and treated with 10-1000ng/ml ciclosporin (Sigma) or 10-1000ng/ml dexamethasone (Sigma) reconstituted in complete DMEM. These concentrations of the immunosuppressive agents were chosen since they have been used by other authors to broadly reflect the concentrations that may be present at the tissue level *in vivo* following transplantation<sup>500-506</sup> and without evidence of toxicity. Cells in fresh medium alone constituted the negative controls. The cells were cultured under these conditions for 2-48 hours. At the end of this time in culture, the supernatants were set aside for later experiments (described in Section 4.3) and the RNA was extracted from the cells using Trizol extraction as described in Chapter 2. cDNA was prepared from the RNA and real-time PCR was used to evaluate *Shh* and *Ptc* expression. Values are expressed as a ratio of the concentration of *Shh* and *Ptc*

mRNA in treated cells relative to untreated (control) cells, where control cells are given a value of 1.

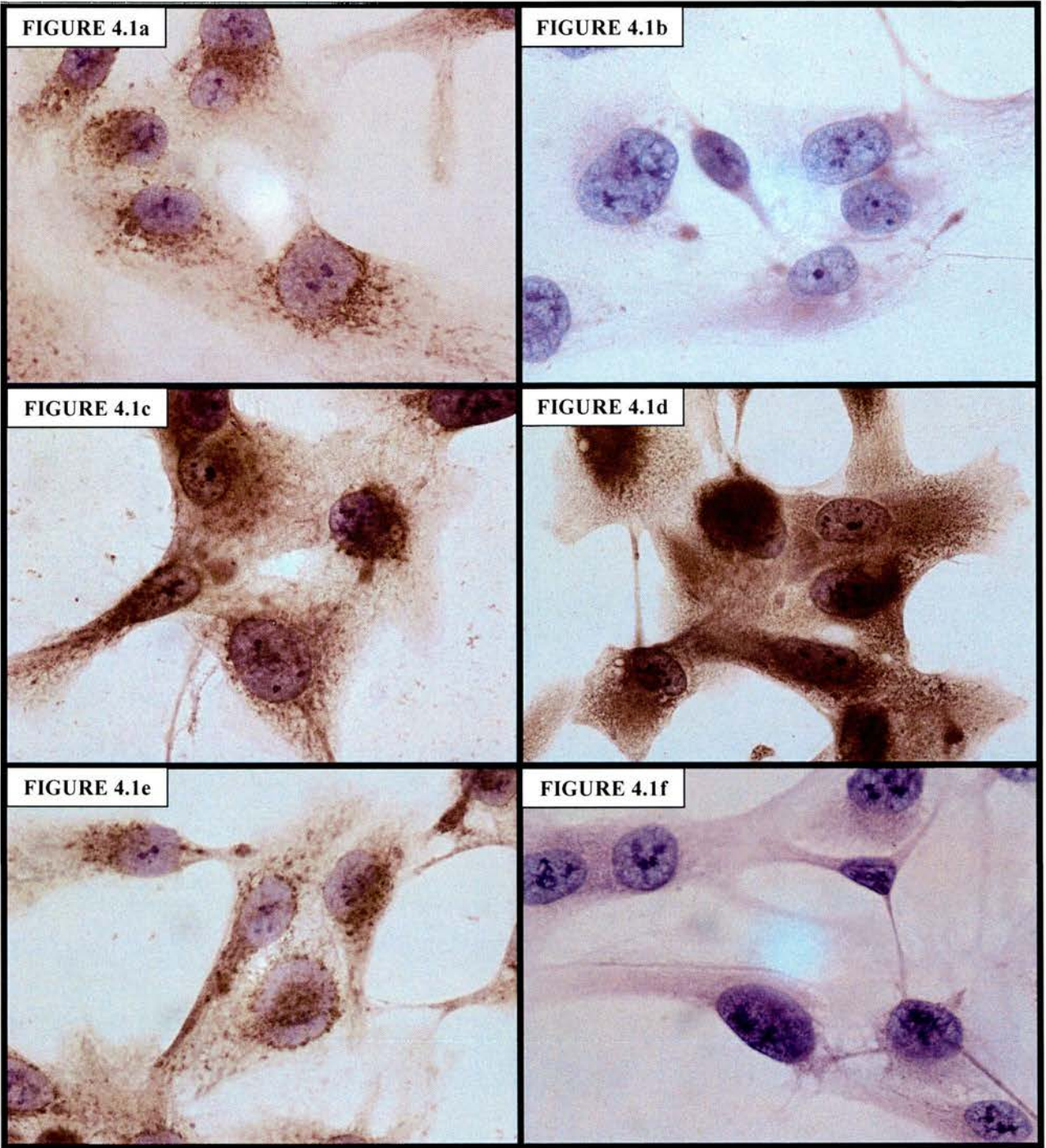
To determine Shh and Ptc protein expression in the ACHN cells, parallel experiments were set up in which glass coverslips, rinsed in alcohol and air-dried, were placed in each well of 6 well culture plates, prior to the addition of ACHN cells. The cells on the coverslips were treated with ciclosporin and dexamethasone and cultured under these conditions for 2-48 hours, as described above. Following this incubation period, the cells were washed with PBS and immunohistochemistry performed on them using polyclonal antibodies to Shh and Ptc (Santa Cruz Biotechnology) to evaluate the expression of these proteins as described in Chapter 2. To check specificity, the same antibodies were pre-incubated with the appropriate specific blocking peptides (Santa Cruz Biotechnology) as outlined in Chapter 2 and applied to coverslips in parallel with experiments in which primary antibodies were applied to the cells. Cells treated in this way served as negative controls for the immunohistochemistry.

The results from these experiments are shown in Figures 4.1 and 4.2. As shown in Figure 4.1, resting ACHN cells express Shh (Figure 4.1a), but not Ptc (Figure 4.1b), protein. In response to ciclosporin, this staining appears to increase in intensity, reaching a maximum at 12 hours (Figure 4.1d) and then declining thereafter. By 48 hours (Figure 4.1e), the Shh expression appeared less intense than in the untreated cells. In contrast, in response to dexamethasone, Shh protein expression did not appear to be modulated (n=3, data not shown).

The results from real-time PCR experiments corroborate these findings. As shown in Figure 4.2, ciclosporin induced an early upregulation in *Shh* expression, preceding the upregulation in protein expression. This effect was maximal at the highest concentration of ciclosporin used (1000ng/ml) and reached a peak at 6 hours. Conversely, although dexamethasone appeared to upregulate Shh expression, the maximum upregulation seen was 1.7 fold, a peak also occurring at 6 hours. The effect induced by dexamethasone, however, did not bear any correlation to the dose

of dexamethasone used. Although it is difficult to determine what constitutes a biologically significant upregulation of mRNA expression, it is generally accepted that a 2-fold or greater increase in gene expression is significant when using real-time PCR<sup>507,508</sup>. This, together with the absence of a dose-dependent effect of dexamethasone, would suggest that the effect of dexamethasone on the ACHN cells may not be significant.



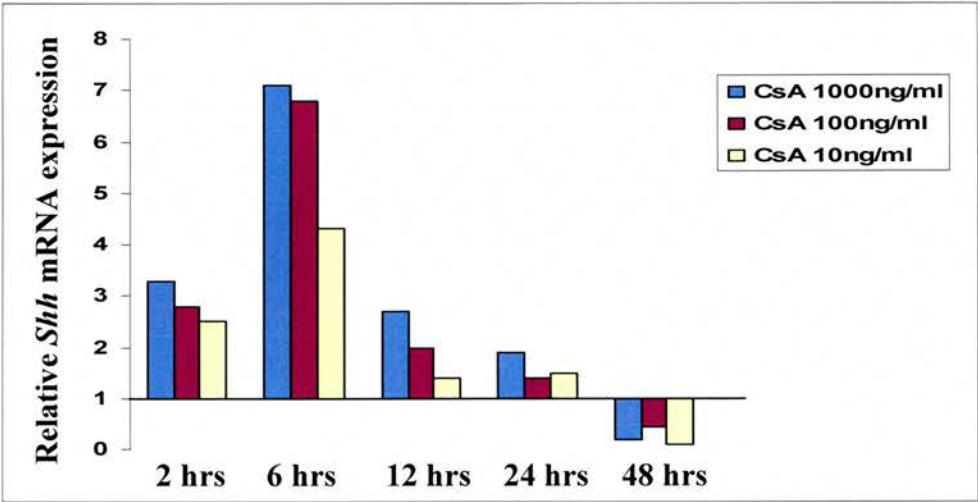


**Figure 4.1**

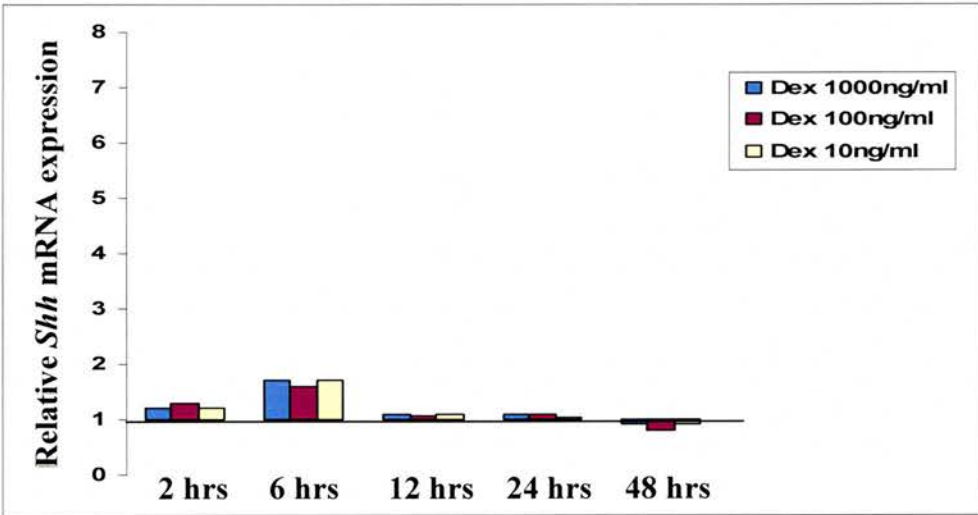
**Shh expression is upregulated by ciclosporin in ACHN cells**

ACHN cells were cultured in medium alone or in the presence of ciclosporin or dexamethasone 10-1000ng/ml for 2 – 48 hours. Immunohistochemistry was performed using polyclonal antibodies to Shh and Ptc, as previously. Untreated ACHN cells expressed Shh (Figure 4.1a, Mag x 1000), but not Ptc (Figure 4.1b, Mag x 1000). In response to ciclosporin 1000ng/ml, intensity of staining increased at 6 hours (Figure 4.1c, Mag x1000), reaching a maximum at 12 hours (Figure 4.1d, Mag x 1000) and, by 48 hours, had decreased dramatically (Figure 4.1e, Mag x 1000). All positive staining could be blocked using the specific blocking peptide (Figure 4.1f, Mag x 1000). Results are representative of 3 individual experiments and data for ciclosporin 1000ng/ml are shown.

**FIGURE 4.2a**



**FIGURE 4.2b**



**Figure 4.2**

***Shh* expression is differentially modulated by ciclosporin and dexamethasone in ACHN cells**

ACHN cells were cultured either in medium alone or in the presence of ciclosporin (CsA) (Figure 4.2a) or dexamethasone (Figure 4.2b) 10-1000ng/ml for 2 – 48 hours. Real-time PCR was then performed on each sample in duplicate using 18s as an internal control to compare the relative expression of *Shh* mRNA in untreated (medium alone) and treated ACHN cells. Values are expressed as a ratio of the concentration of *Shh* mRNA in treated cells relative to untreated cells, where the latter are given a value of 1. The results presented are representative of 3 independent experiments.

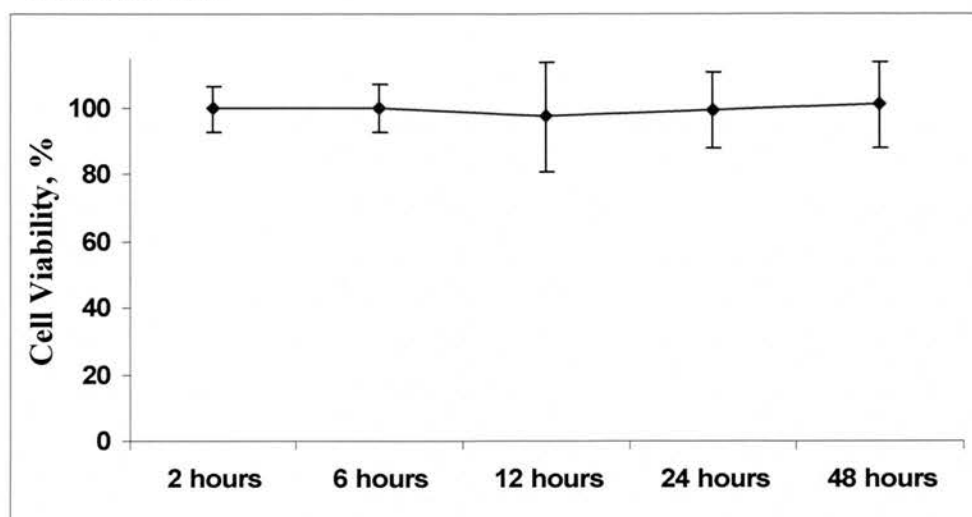
#### 4.2.2 Evaluating the toxicity of ciclosporin and dexamethasone to ACHN renal epithelial cells

Although ciclosporin and dexamethasone have been used in *in vitro* experiments previously at the concentrations used here without evidence of toxicity, it was important to evaluate whether these agents were toxic to the ACHN cells used in these experiments. Thus, to confirm that neither agent was toxic to the cells used in my system, 3 methods were used to assess cell viability. Firstly, the cells were inspected by light microscopy to determine the presence of any gross changes in cell morphology. Secondly, cell viability was assessed by exclusion of trypan blue entry<sup>501</sup>. Finally MTT assays were performed, as described in Chapter 2, to determine whether ciclosporin and dexamethasone in the concentration ranges used were toxic to the ACHN cells. The MTT assay allows an assessment of cell viability since only metabolically active cells are capable of reducing the MTT tetrazolium compound into a coloured formazan product<sup>509</sup>. The quantity of formazan product is then measured by the amount of absorbance at 570nm which is proportional to the number of living cells in culture.

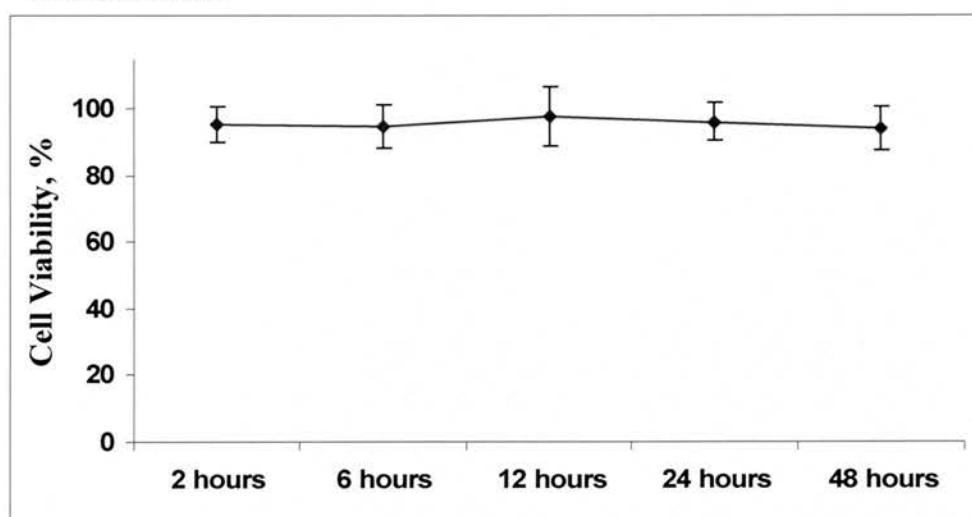
Figure 4.3 illustrates the results from the MTT assay. Consistent with previous reports in the literature, the results from the assay demonstrated that neither ciclosporin (Figure 4.3a) nor dexamethasone (Figure 4.3b) were toxic to the ACHN cell line at any of the concentrations used. Representative results at the highest concentration of each agent (1000ng/ml) are shown.



**FIGURE 4.3a**



**FIGURE 4.3b**



**FIGURE 4.3**

**Evaluation of ciclosporin and dexamethasone cytotoxicity on ACHN cells using MTT**

ACHN cells were incubated with either ciclosporin or dexamethasone in the concentration range 10-1000ng/ml for 2-48 hours. Viability was evaluated using the MTT assay. Data for 1000ng/ml ciclosporin (Figure 4.3a) and dexamethasone (Figure 4.3b) are shown. Results are presented as the ratio of treated to untreated cells as a percentage where untreated cells are given a value of 100%. Experiments were performed in triplicate and means and standard deviations are shown.

### 4.3 Shh release from ACHN cells

Given that ciclosporin induced a marked and early upregulation in *Shh* mRNA expression and that this appeared to be mirrored by changes in protein expression resulting in the apparent loss of protein expression detected by immunohistochemistry at later time points, it was tempting to speculate that Shh may be released from the ACHN cells in response to ciclosporin treatment. Few antibodies and reagents are available for investigation of the Shh signalling pathway and this ensures that attempts to detect Shh release are difficult. It is only relatively recently that a murine Shh ELISA has become commercially available. However, to address the possibility that ciclosporin might induce Shh release from ACHN cells, 2 approaches were employed, ELISA and Western blotting techniques.

#### 4.3.1 ELISA

Although a human Shh ELISA is not yet commercially available, an attempt was made to use the commercially available murine Shh ELISA (R&D Systems) for the detection of human Shh. Human and murine Shh differ in only one amino acid<sup>2</sup> and most of the commercially available antibodies are capable of recognising Shh from more than one species. Furthermore, recombinant murine Shh has been used in the literature for the investigation of human cell responses<sup>20,65,510</sup>. Thus, it was possible that the murine Shh ELISA available may also recognise human Shh. The murine sandwich ELISA utilises a rat anti-mouse capture antibody for primary Shh detection and a biotinylated rat anti-mouse secondary antibody and is calibrated against the same recombinant murine Shh utilised in this work and other human studies in the literature<sup>20,510,511</sup>. The ELISA was therefore used initially to determine whether Shh could be detected in the cell culture supernatant of cells treated with the immunosuppressive agents ciclosporin and dexamethasone. The ELISA was performed according to the manufacturer's instructions and supernatants from the experiments described in Section 4.2.1 were used.

Although the recombinant Shh standards produced a robust standard curve, Shh failed to be detected in supernatants from either the control cells or the ciclosporin- or dexamethasone-treated cells. These results suggest that either the ELISA is failing to detect human tissue-derived Shh, that Shh is not released into the supernatant in response to treatment with the immunosuppressive agents or that Shh is released but in a form not recognised by the ELISA.

#### **4.3.2 Western blotting**

That Shh was not detected in the cell culture supernatants may well reflect the inability of the murine ELISA to detect human Shh and, indeed, the reported cross-reactivity of the ELISA for human Shh is in the order of 11.8%. Thus, a different approach was required. To explore possible release of Shh by the ACHN cells, Western blotting techniques were employed. The aim was to determine whether Shh protein expression in untreated cells could be detected by Western blotting; whether this expression was modulated by treatment with ciclosporin or dexamethasone and finally, if expression in the cells was modulated, whether this was associated with the appearance of Shh protein in the supernatant.

##### *4.3.2.1 Shh protein is detected in ACHN cells by Western blotting*

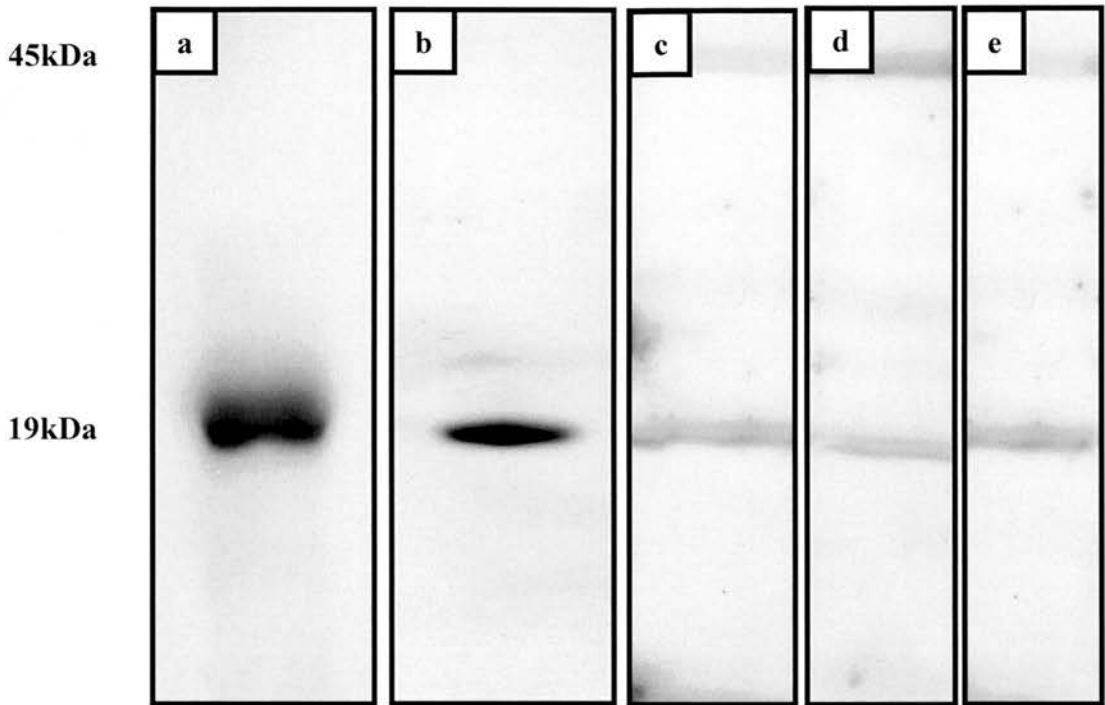
To determine whether Shh protein could be detected by Western blotting techniques, ACHN cells were sonicated in protein lysis buffer as described in Chapter 2. Protein quantification was undertaken using the Bradford protein assay (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) and each sample was assayed in triplicate. 100µg of each protein sample was used in the Western blotting experiments. Western blot protocols have been described in more detail in Chapter 2. Briefly, protein samples were added to 2x protein loading buffer, heated at 100°C for 10 minutes, and electrophoresed at 100V for 2 hours alongside pre-stained molecular weight markers (Invitrogen, Paisley, UK). Lysates from untreated ACHN cells and cells treated with 10-1000ng/ml ciclosporin or dexamethasone were evaluated alongside 100ng/ml rShh which served as the positive control. Following transfer to nitrocellulose at

150V for 30 minutes, the membranes were incubated overnight in blocking buffer to block non-specific binding. The membranes were then probed for 2 hours with either a goat polyclonal antibody to the N-terminus of Shh (Santa Cruz Biotechnology) or a mouse monoclonal antibody to Shh, 5E1 (Developmental Studies Hybridoma Bank). In a parallel experiment, to confirm the antibody specificity of the goat polyclonal antibody, an aliquot of the antibody was pre-incubated with the specific blocking peptide (Santa Cruz Biotechnology) prior to application to the membrane. The secondary antibodies used were HRP-rabbit anti-goat and HRP-rabbit anti-mouse and were applied for 2 hours at 1:2000 dilution. Detection was effected using the ECL plus system (Amersham Pharmacia Biotech, Amersham, UK). Excess reagent was removed and the membrane placed under X-ray sensitive film for various lengths of exposure time (30 seconds – 10 minutes).

Representative results are shown in Figure 4.4. Although both the goat polyclonal antibody (Figure 4.4a) and the 5E1 mouse monoclonal antibody (Figure 4.4b) clearly detected the rShh used as a positive control, only the goat polyclonal antibody was able to detect Shh in the ACHN lysate (Figure 4.4c), despite numerous protocol optimisation strategies. Two relevant bands were detected in the ACHN cell line, one at 45kDa and one at 19kDa consistent with the presence of full-length precursor Shh and its active cleaved form respectively (Figure 4.4c). These bands could be blocked when the membranes were incubated with an antibody/blocking peptide cocktail, thus suggesting that the bands were specific in nature. However, in response to ciclosporin or dexamethasone, there was no apparent change in Shh expression in the treated compared to the untreated cells (Figure 4.4d and e).



**FIGURE 4.4**



**FIGURE 4.4**

**Western blots of ACHN cell lysates**

Lysates from untreated cells (medium alone) and cells treated with either 10-1000ng/ml ciclosporin or dexamethasone were evaluated using Western blotting. Both a goat polyclonal and mouse monoclonal antibody to Shh were used. Although both the goat polyclonal (Figure 4.4a) and the mouse monoclonal (5E1) (Figure 4.4b) antibodies detected 100ng rShh used as the positive control, only the goat polyclonal antibody detected Shh in the ACHN (untreated, control) lysate (Figure 4.4c). However, in response to ciclosporin or dexamethasone, there was no apparent change in Shh expression in the treated cells. Figures 4.4d and 4.4e represent the effect of treatment with 1000ng/ml ciclosporin and dexamethasone respectively.

#### 4.3.2.2 *Shh is not detected in the supernatant of cells treated with ciclosporin or dexamethasone*

Despite the difficulties associated with optimisation of the Western blot experiments, attempts were also made to detect Shh protein in the supernatant of untreated and ciclosporin or dexamethasone-treated cells. ACHN cells were seeded into 162cm<sup>2</sup> flasks and grown to 75% confluence as in the previous experiments. The cells were treated with either medium alone, ciclosporin or dexamethasone in the concentration range 10-1000ng/ml in a volume of 10mls. The supernatants were harvested following 2-48 hour incubations. Each supernatant was transferred to dialysis tubing and placed on polyethylene glycol (PEG) (Fisher Scientific UK, Loughborough, Leics, UK), the latter binding water and thus concentrating the protein to a volume of less than 2.5mls. The protein content was quantified, as previously, using the Bradford protein assay, performed on each sample in triplicate. 100µg of protein was analysed by Western blot as described above using the goat polyclonal antibody to the N-terminus of Shh as the primary antibody (Santa Cruz Biotechnology).

Using this technique, although the positive control rShh was readily detected, the presence of Shh in the cell supernatants could not be demonstrated.

## 4.4 Primary murine renal epithelial cell cultures

From the results so far presented, it would appear that ciclosporin is capable of upregulating *Shh* mRNA in the ACHN cell line and that this may be mirrored at the protein level as reflected by the immunohistochemistry results. Conversely, dexamethasone did not significantly modulate either *Shh* mRNA or Shh protein expression. The peak in Shh protein expression induced by ciclosporin is followed by an apparent decline in expression. This observation would be consistent with either a release of Shh protein from the ACHN cells or intracellular modification resulting in the masking of Shh protein to the detection antibody. Given that the recently proposed targets for Shh include immune cells<sup>20,21,149</sup> and fibroblasts<sup>102</sup>, it is tempting to speculate that Shh may be released from epithelium whereupon it can act on these cells. However, the methods used above to investigate the possibility of Shh release were largely unsuccessful and are hampered by the scarcity of commercially available reagents. Thus, it remains unclear whether these agents induce the release of Shh from epithelium. Furthermore, although the results from the ACHN cell line are interesting, it is not clear whether these findings adequately represent the situation in normal renal tissue or *in vivo*. Although the ACHN cell line has been previously used to represent normal proximal tubular epithelium, this is probably not appropriate. The ACHN cell line is derived from a renal adenocarcinoma and, as such, does not therefore represent normal renal epithelium. This may be of particular importance in the context of Shh signalling. Although significant Hh expression is not detected in many adult tissues, recent reports have suggested that active Hh signalling may be critical to the growth of a number of tumour cell lines<sup>66</sup>. For example, although Hh signalling appears to be important in the growth of oesophageal tumours, as indicated by cell line studies, there have been no reports of Shh expression in the adult oesophagus<sup>66</sup>. Thus, results suggesting an effect on Shh expression in a tumour cell line require to be interpreted with caution. Therefore, a new approach was required to fulfil 2 roles. Firstly, the approach must satisfy the need to investigate normal renal epithelium, rather than extrapolating results from a cell line, and secondly, in view of the difficulties encountered in the investigation of Shh release from human epithelium, it was necessary to employ a murine model

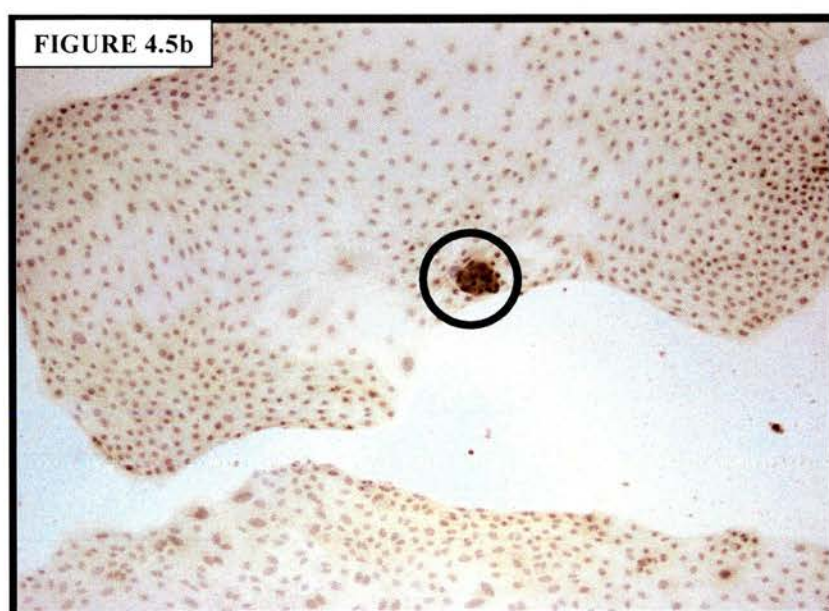
since there is, at least, a commercially available Shh ELISA available to allow Shh protein quantification. In this regard, primary cultures of murine renal epithelium were prepared and investigated for both the expression of Shh and the possible release of Shh protein in response to treatment with ciclosporin.

#### **4.4.1 Primary renal epithelial cell (REC) cultures**

Primary epithelial cultures were prepared from C57BL/6 (B6) mice by collagenase digestion of renal cortex based on methods described previously<sup>512,513</sup> and outlined in Chapter 2. Following digestion and precipitation on ice, the tubules were resuspended in complete DMEM F12 culture medium and plated into 6 well culture plates. To minimise the outgrowth of fibroblasts, the primary cultures were maintained in serum-free conditions. The tubules from one pair of kidneys were distributed between four 6-well plates, with 2mls of medium in each well. The cells were cultured for 10 days and the medium was replaced with fresh DMEM F12 medium every 2 days.

By the end of this incubation period, large islands of confluent cells were apparent with defined advancing edges. Although the culture conditions promote the growth of proximal tubular epithelial cells<sup>512,514,515</sup>, the islands were observed to consist of markedly heterogeneous populations of cells. In order to define the phenotype of the cells prior to investigating Shh expression and the effect of immunosuppressive agents on the cells, experiments were set up in which glass coverslips were placed at the bases of the wells in 6 well plates. Cells cultured on these glass coverslips were employed to immunohistochemically investigate the phenotype of the cells cultured. Following washes to remove culture medium, the cells were probed with monoclonal anti-pancytokeratin and -vimentin antibodies (Sigma) to broadly identify epithelial and fibroblastic phenotypes within the cell culture islands as described previously<sup>501</sup>. The antibodies were incubated at 1:100 dilution for 2 hours and secondary detection performed using a biotinylated rabbit anti-mouse antibody for 1 hour. As previously, positive signalling was identified using diaminobenzidine (Dako Ltd).

As shown in Figure 4.5a, most cells present in the cell culture islands were pancytokeratin positive suggesting that the primary culture was of renal epithelial phenotype. However, there were present smaller collections of cells which were vimentin positive (Figure 4.5b) suggesting that a few non-epithelial, probably fibroblastic, components were present.



**FIGURE 4.5**

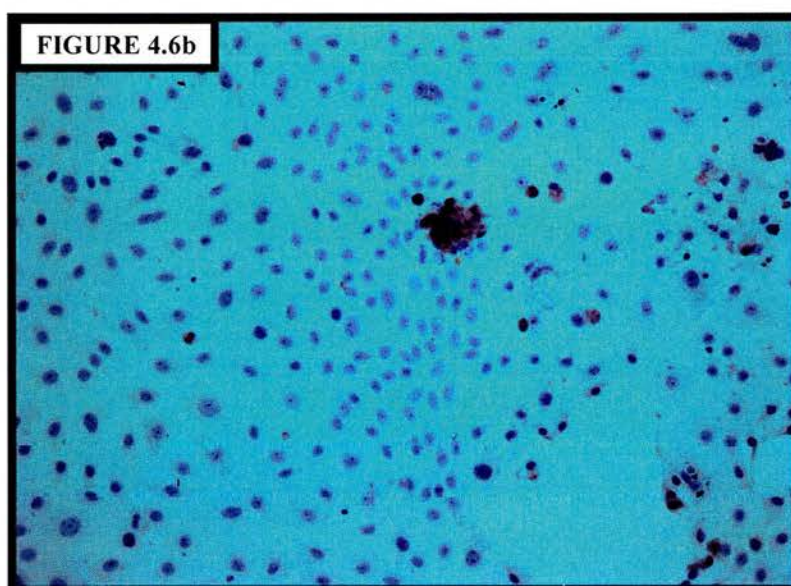
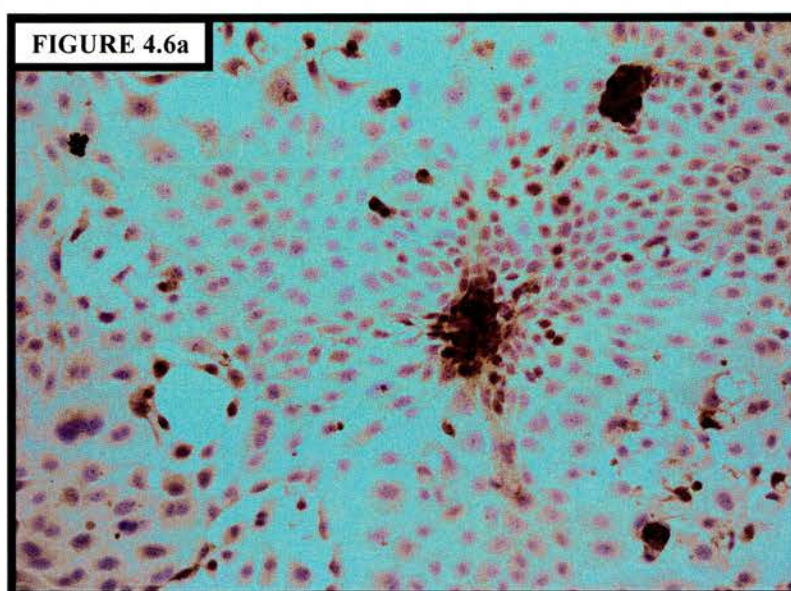
**Immunohistochemical phentotyping of Renal Epithelial Cell (REC) cultures**

RECs were prepared from C57BL/6 murine kidneys by collagenase digestion. Immunohistochemistry was performed using antibodies to pancytokeratin (Figure 4.5a, Mag x 200) and vimentin (Figure 4.5b, Mag x 200). As shown, most cells present were pancytokeratin positive. Smaller clusters of vimentin-positive cells (circled) were also evident.



#### 4.4.2 Shh and Ptc expression in REC cultures

Having determined that most cells present in the cell cultures were of epithelial phenotype, immunohistochemistry was performed to determine whether the RECs express Shh and Ptc. Representative results are illustrated in Figure 4.6. It can be seen that, using the goat polyclonal antibody to the N-terminus of Shh at the same dilution as that used previously in this work (1:40), the epithelial cells stained weakly for Shh. This staining could be blocked effectively using the specific blocking peptide for the Shh antibody. Conversely, small clusters of cells, in keeping with those staining positively for vimentin, expressed Shh relatively strongly at 1:40 (Figure 4.6a). When investigated for the expression of Ptc, the pancytokeratin positive cells were negative for Ptc (Figure 4.6b). In contrast, the vimentin-positive cells were also Ptc-positive (Figure 4.6b). That fibroblasts are positive for Ptc in this work is consistent with previous reports in which fibroblasts were found to express Ptc and were capable of responding to a Shh signal<sup>102</sup>.



**FIGURE 4.6**

**Renal Epithelial Cells express Shh, but not Ptc**

RECs were investigated for the expression of Shh and Ptc using immunohistochemistry. RECs were observed to stain weakly for Shh with the most marked expression in those cells previously observed to be vimentin-positive (Figure 4.6a, Mag x 200). Conversely, Ptc expression was absent in all but these clusters of cells (Figure 4.6b, Mag x 200) consistent with the expression of Ptc by fibroblast-type rather than epithelial cells.

## **4.5 Shh expression by and release from REC cultures in response to ciclosporin and dexamethasone**

Using RECs prepared as outlined above, the cells were treated with ciclosporin and dexamethasone in similar experiments to those described above in Section 4.1.

Briefly, RECs were cultured in the presence of 10-1000ng/ml of either ciclosporin or dexamethasone for 2-48 hours. At the end of this period in culture, the supernatants were harvested and the murine Shh ELISA (R&D Systems) was employed to detect any Shh in the supernatant, according to the manufacturer's instructions. RNA was also extracted from the cells using the Trizol extraction techniques described in Chapter 2. Following reverse transcription of the RNA to cDNA, standard RT-PCR and real-time PCR were employed using the primers outlined in Tables 2 and 3 in an attempt to determine whether *Shh* expression is upregulated in response to treatment with ciclosporin or dexamethasone.

To determine Shh and Ptc protein expression in the RECs, parallel experiments were performed in which glass coverslips, rinsed in alcohol and air-dried, were placed in each well of 6 well culture plates, prior to the addition of cells. The cells were treated with 10-1000ng/ml ciclosporin and dexamethasone and cultured under these conditions for 2-48 hours as described above. Following this incubation period, the cells were washed with PBS and immunohistochemistry performed on the cells using polyclonal antibodies to Shh and Ptc (Santa Cruz Biotechnology) to evaluate the expression of these proteins.

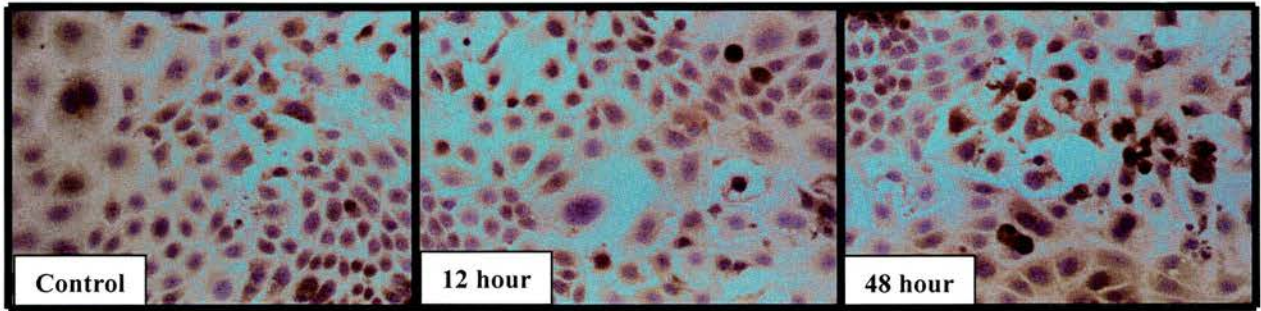
Further parallel experiments were set up to allow analysis of possible toxic effects of the immunosuppressive agents on the RECs using the MTT assay, as performed for the ACHN cells above. As in the ACHN cell culture system, the MTT assay confirmed that neither ciclosporin nor dexamethasone in the dose range 10-1000ng/ml were toxic to the RECs.

Although rShh, used as a positive control, was detected by the Shh ELISA, the ELISA failed to detect any Shh in the cell culture supernatants. Furthermore, in contrast to the results seen using the ACHN cell line, neither ciclosporin nor dexamethasone treatment modulated the Shh protein expression of RECs detected using immunohistochemistry (Figure 4.7).

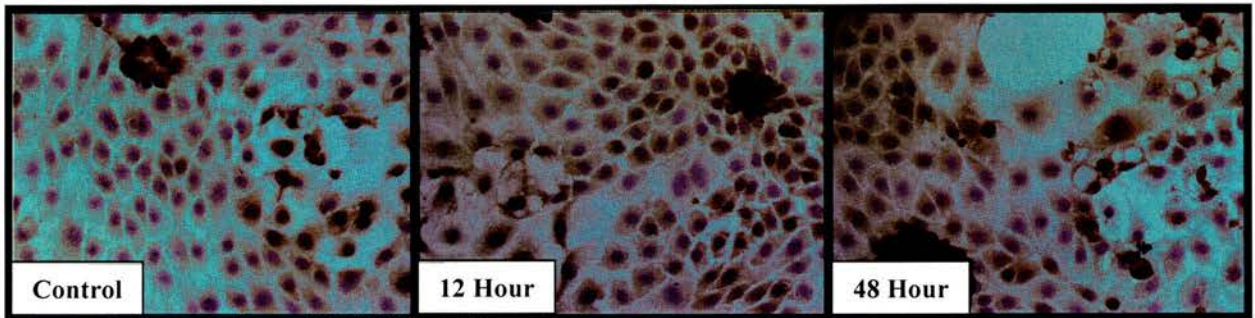
Furthermore, despite obtaining good quality RNA, *Shh* expression could not be detected in any of the REC samples, irrespective of which primers were used and whether RT-PCR or real-time PCR techniques were employed. The primers used included sequences previously employed by our group to detect *Shh* using RT-PCR and real-time PCR and are documented in Chapter 2. Despite considerable optimisation (altering magnesium concentrations, dNTPs and the annealing temperatures for the RT-PCR primers), *Shh* expression was detected neither in the REC samples nor indeed in both archival and freshly prepared murine thymus utilised previously as a positive control.



**FIGURE 4.7a**



**FIGURE 4.7b**



**FIGURE 4.7**

**The expression of Shh in Renal Epithelial Cells is not modulated by ciclosporin or dexamethasone**

Following treatment of RECs with 10-1000ng/ml ciclosporin or dexamethasone, immunohistochemistry was used to evaluate Shh expression as previously. Neither ciclosporin nor dexamethasone modulated the expression of Shh in RECs. Illustrated are untreated controls together with the expression pattern seen in response to 1000ng/ml ciclosporin (Figure 4.7a) and 1000ng/ml dexamethasone (Figure 4.7b) following 12 and 48 hours in culture.

## 4.6 Damage fails to modulate Shh release from RECs

It has been hypothesised that Hh signalling may be important in the adult during inflammation, repair and/or remodelling processes<sup>149,153,161</sup>. Shh is expressed in a variety of epithelia including pulmonary, gut and thymus<sup>67,147-149,516</sup> and may be upregulated at sites of inflammation<sup>149,161</sup>. It is conceivable then that Shh may be released at sites of epithelial damage whereupon it has the potential to act in a paracrine fashion on other cells types including cells of the immune system<sup>20,21</sup>, fibroblasts<sup>102</sup> and endothelial cells<sup>65</sup>, thus perpetuating the inflammatory response. Indeed both T cells and macrophages express Ptc and may be able to respond to a Shh signal<sup>20,21,149</sup>. Given that ciclosporin upregulated Shh mRNA expression in ACHN cells, it was tempting to speculate that ciclosporin may induce Shh release from epithelium. From the limited data available, ciclosporin did not appear to induce the release of Shh from epithelium. In light of these results, the aim of the following section of work was to perform preliminary experiments to determine whether established methods of inducing cellular damage could induce Shh expression or release from tubular epithelium. In order to pursue this line of enquiry, 2 damaging methods were employed: oxidative cell damage and ultraviolet B irradiation-induced damage.

### 4.6.1 Oxidative cell damage

Hydrogen peroxide may be used in *in vitro* cultures to induce oxidative cell damage. It has been shown to cause a rapid increase in intracellular free calcium concentration in several cell types including renal tubular cells<sup>517,518</sup>, enhanced calcium influx into mitochondria and the disruption of mitochondrial metabolism leading to cell death<sup>519,520</sup>. Previous reports in the literature using hydrogen peroxide to induce oxidative cell damage have used a range of concentrations in tubular epithelial cultures, from 0-2mM<sup>521-523</sup> for incubation periods of 2-48 hours.



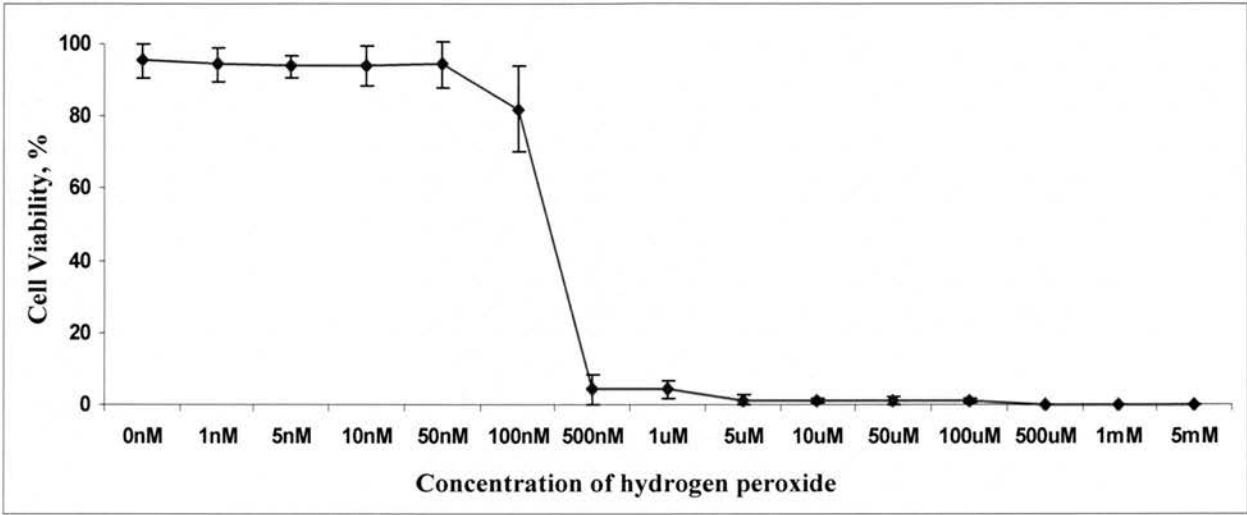
Therefore, in this series of experiments, RECs were prepared, as previously, and plated into 6 well culture plates. After 10 days in culture, when large confluent islands of cells were apparent, the medium was replaced with hydrogen peroxide (Sigma) in the concentration range 0-5mM and the cells were incubated for 1-24 hours. After this incubation period, the cells were washed and the medium was replaced with fresh medium. At the higher end of the concentration range of hydrogen peroxide used, notably 5 $\mu$ M – 5mM, the cells had become detached by the end of the 24 hour period in culture and required to be centrifuged for 5 minutes at 100g prior to re-suspension in fresh medium. The cells were incubated at the end of a further hour in culture with fresh medium to allow any Shh present in the damaged cells to be released into the culture medium. The supernatants were harvested and set aside for later analysis using the murine Shh ELISA (R&D Systems). In a parallel series of experiments, following incubation with the hydrogen peroxide, the supernatants were drawn directly from the culture wells without prior cell washing and replacement of the cell medium. This was performed in order to avoid missing any Shh released early into the culture medium that would have been removed by the subsequent washes. Supernatants harvested from both experiments were analysed using the murine Shh ELISA (R&D Systems) according to the manufacturer's instructions.

In a further set of parallel experiments, the MTT assay was used, as described in Chapter 2, to assess cell viability at the end of the incubation period. As described above, when cell detachment was apparent in the cultures, the cells were first centrifuged for 5 minutes at 100g prior to removal of the medium and replacement with fresh medium and MTT reagent. An example of the results obtained following a 2 hour incubation with varying concentrations of hydrogen peroxide is demonstrated in Figure 4.8. As shown, increasing concentrations of hydrogen peroxide are associated with decreasing cell viability in the REC cultures.

Although a reduction in cell viability was clearly seen in response to treatment with hydrogen peroxide, Shh failed to be detected by ELISA in any of the cell culture

supernatants examined, irrespective of whether supernatants were harvested following a wash with medium following removal of the hydrogen peroxide or whether the supernatants were harvested directly from the hydrogen peroxide incubation. The rShh used as a positive control was however detected by the Shh ELISA in all experiments.

**FIGURE 4.8**



**FIGURE 4.8**

**Evaluation of hydrogen peroxide cytotoxicity on RECs using MTT**

Renal epithelial cells were incubated with hydrogen peroxide in the concentration range 1nM-5mM for 1-24 hours. Viability was evaluated using the MTT assay. Representative data for the 2 hour incubation period are shown. Results are presented as the ratio of treated to untreated cells as a percentage where untreated cells are given a value of 100%. Experiments were performed in triplicate and means and standard deviations are shown.

#### 4.6.2 Ultraviolet B irradiation-induced damage

The results outlined above in Section 4.4.1 were unexpected. If indeed Shh is released from any damaged epithelium, it might have been anticipated that oxidative cell damage would have resulted in Shh being released into the culture medium. Indeed, a wide concentration range of hydrogen peroxide was employed and cytotoxicity clearly demonstrated using the MTT assay. That early peaks in Shh release may have been overlooked on account of the subsequent washing was circumvented by separate experiments in which washing was omitted. In neither series of experiments was Shh detected. There are a number of reasons why this might have been the case, outwith the possibility that Shh may not be released in response to oxidative cell damage. Notably, either the overt introduction of hydrogen peroxide into the ELISA system, or indeed any carryover that may have resulted following cell washing, carries with it the strong possibility of interference with the performance of the ELISA, a point of particular relevance given that the ELISA employs a streptavidin-horseradish-peroxidase (HRP) conjugate. Indeed, such an effect is likely to result in any system in which a cytotoxic agent is utilised to induce damage. This situation cannot be readily circumvented since thorough washing to exclude possible carryover effects inevitably will remove protein released in the early phases of cell damage, thus preventing its detection by the ELISA. Therefore, in the following series of experiments, a different method of cell damage was employed which avoided the use of cytotoxic agents.

Ultraviolet B (UVB) irradiation has been shown to induce apoptosis in a range of cell types. These include mast cells<sup>524</sup>, epithelial cells<sup>525-527</sup>, macrophages<sup>528</sup> and fibroblasts<sup>529</sup> and protocols used previously have included treatments of 10mJ/cm<sup>2</sup>-500mJ/cm<sup>2</sup> for durations of 30 minutes to 4 hours to induce apoptotic changes.

In 3 preliminary experiments, RECs were prepared as previously and subjected to high levels of UVB (100mJ/cm<sup>2</sup> – 500mJ/cm<sup>2</sup>) for 1 hour. Supernatants were removed from the cells both to treatment, at the termination of treatment and 1, 6, 12 and 24 hours after the termination of treatment. Twelve hours following the

termination of treatment, the cells were only weakly adherent and macroscopically unhealthy with condensed nuclei. By 24 hours, all cells had become detached from the base of the culture wells and Trypan blue exclusion revealed >95% cell death.

The culture supernatants removed were analysed as previously by murine Shh ELISA (R&D Systems). As in section 4.6.1 above, although the rShh used as a positive control was detected by the Shh ELISA, Shh was not detected in any of the cell culture supernatants tested.

## 4.7 Discussion

The work in this chapter has investigated, using both a human tubular cell line (ACHN) and primary cultures of murine renal epithelial cells, factors that may influence whether Shh is expressed and/or released from renal tubular epithelium. These are interesting areas for a number of reasons. Firstly, Shh expression is upregulated at sites of epithelial damage suggesting that Hh signalling may be important in repair and/or remodelling responses<sup>149</sup>. Secondly, several reports in the literature have identified peripheral cellular targets of Shh signalling including cells of the peripheral immune system<sup>20,21</sup>, endothelial cells<sup>65</sup> and fibroblasts<sup>102</sup>. Taken together, these reports indicate that the epithelium and these responder cells have the potential to interact at sites of renal epithelial injury or disease. Thus, further delineation of factors influencing Shh expression and identifying whether Shh release occurs in Shh-expressing cells in the kidney may help determine a role for Shh, either in the normal kidney, or in the kidney damaged by disease.

It is important to note at this stage that one of the key factors hampering the experiments described in this chapter was the lack of robust reagents for investigation of the Shh signalling pathway. As described, although it was possible to detect human *Shh* mRNA, it was not possible to directly measure human Shh protein except through the use of immunohistochemistry. In the murine studies, evaluation of Shh protein was possible, albeit that Shh failed to be detected, through the use of the commercially available murine Shh ELISA. However, attempts to quantify murine *Shh* mRNA expression were hampered by the inability to optimise the RT-PCR primers and real-time primers and probes to allow qualitative and quantitative studies to be performed. Together, these difficulties ensured that attempts to link together changes in mRNA expression quantitatively with changes in protein release or expression were made very difficult. Given these methodological difficulties, it is important to interpret any findings presented with caution since their relevance can not be fully delineated in the absence of good reagents to fully evaluate Shh expression at both the protein and mRNA level.



#### **4.7.1 The effect of immunosuppressive agents on Shh expression in renal tubular epithelium**

The experiments in the first part of this chapter were designed to evaluate whether the immunosuppressive agents ciclosporin and dexamethasone altered the expression of Shh in the renal epithelial cells. Given the findings from Chapter 3 suggesting that proximal tubular epithelial cells were the sites of Shh expression in normal adult human kidney, the renal adenocarcinoma cell line, ACHN, an *in vitro* model of tubular epithelium, was initially used to evaluate the effect of the ciclosporin and dexamethasone on Shh expression in these cells. The experiments were also performed using primary cultures of murine renal epithelial cells. The effects of these agents on both mRNA and protein expression were examined. Furthermore, the possibility that Shh release may be induced by treatment with these agents was explored in both systems.

Taken together, the results showed a marked upregulation of Shh mRNA and protein expression in response to ciclosporin, but not dexamethasone, in ACHN cells. Conversely, in RECs, such upregulation of Shh protein expression in response to ciclosporin was not apparent. It is unfortunate that real-time PCR could not be utilised to investigate the expression of *Shh* mRNA in response to ciclosporin and dexamethasone in the RECs as utilised previously during investigation of the ACHN cell line. Indeed, neither real-time PCR nor standard RT-PCR detected *Shh* expression in the REC samples. The reasons for this inability to evaluate mRNA expression are not readily apparent. The RNA derived from the cells was of good quality as determined using the RNA quality check gels described in Chapter 2. Furthermore,  $\beta$ -actin could be readily detected in the samples suggesting that the problem lay in the nature of the primers. Indeed, the murine thymus cDNA previously utilised as a positive control<sup>21</sup> failed to demonstrate *Shh* mRNA expression on repeated evaluation using various primer sets. This remained true irrespective of the method of RNA extraction employed, the age of the primers employed, the concentrations of magnesium and dNTPs used, the temperature of the

reactions and the investigator performing the experiment. Thus, further evaluation of the effect of ciclosporin and dexamethasone relies purely on results obtained using immunohistochemical detection of protein expression. Using this technique and accepting that it is a qualitative rather than a quantitative method of evaluating protein expression, it is interesting that, in contrast to the apparently upregulated expression of Shh protein seen in ACHN cells in response to ciclosporin, the same effect was not observed in the RECs. Rather, neither ciclosporin nor dexamethasone induced a change in the apparent intensity of staining. Thus, there is the possibility that the ACHN cells and the RECs do not behave similarly in response to ciclosporin treatment.

If indeed the RECs do not respond to ciclosporin in the same way as the ACHN cell line, it is interesting to speculate why not. The primary murine renal epithelial cell cultures described here were cultured under conditions reported to promote a proximal tubular cell phenotype<sup>512,514,515</sup>. Nevertheless, cultures produced very heterogeneous populations of cells, which were phenotypically either pancytokeratin positive or vimentin positive i.e. broadly epithelial or fibroblastic in nature. What cannot be verified is whether all the epithelial cells were proximal tubular cells. Tubular epithelial cells are characterised as being keratin positive, smooth muscle actin negative, desmin negative, and vimentin negative. However, differentiating proximal from distal tubular cells can be difficult. Several phenotypic and functional characteristics of proximal tubular cells have been described<sup>530</sup> and used to differentiate these cells from other renal epithelial cells. These include the cell surface markers alkaline phosphatase<sup>531</sup>, dipeptidyl peptidase IV and gamma-glutamyl transpeptidase, an apical brush border enzyme<sup>532</sup>, and also functional markers such as sodium-dependent glucose transport and cAMP production induced by parathyroid hormone, but not vasopressin and gluconeogenesis<sup>530</sup>.

In the experiments described in this chapter, extensive phenotyping has not been performed to confirm that all epithelial cells are derived from the PCT. Indeed, it is possible that, although the cells were cultured under conditions believed to promote the growth of proximal tubular cells, the cultures may not be cultures of pure PCT

cells. Nevertheless, given that Shh was expressed by the RECs albeit with less intense staining than in the tissue sections, together with the pancytokeratin and vimentin positive and negative expression respectively, suggests that at least the majority were likely to be proximal tubular cells. Nevertheless, the RECs failed to respond to ciclosporin in the same way as the ACHN cells. This may reflect a disadvantage of primary cultures of PCT cells, since both the loss of PCT markers and acquisition of new markers has been described in these cells under culture conditions<sup>530</sup>. Alternatively, the ACHN cells might respond in a manner not typical of the RECs.

In the context of Shh signalling, this latter issue may be important – Hh signalling has been shown to be important in the initiation, growth and survival of medulloblastomas, small cell lung cancer and pancreatic adenocarcinoma<sup>533,534</sup>. More recently Shh expression has also been demonstrated in a large number of cell lines many of which are derived from tissues not known to be associated with active Shh signalling *in vivo*<sup>66,533-535</sup>. Furthermore, that the ACHN cell line differs from normal renal epithelium in terms of cytokine or receptor profiles and antigen expression has been reported<sup>536-539</sup>. Thus, employing a cancer cell line for investigating Shh pathway components is probably not the most ideal system to employ, particularly when active cell signalling can contribute to cell growth<sup>66</sup>. Other cell lines which might have been better to use include those derived from normal (Madin-Darby canine kidney cells) or embryonic (HK293 cells), although use of the former is complicated by the lack of canine reagents for investigation of the Shh pathway. HK293 cells derived from human embryonic cortex or, indeed, pre-plated human renal cortical cells or cryopreserved human renal proximal tubules which are now available commercially (In Vitro Technologies, Baltimore, Maryland, USA) may have been better alternatives.

Even if the effect of ciclosporin on Shh expression is limited to an effect on the ACHN cell line, this result is interesting and appears to a specific response to ciclosporin since it was not demonstrated when cells were treated with dexamethasone. Ciclosporin is a cyclic decapeptide derived from the soil fungus

*Tolypocladium inflatum*. In the peripheral T cell, ciclosporin blocks proliferation by inhibiting the phosphatase activity of the calcium activated enzyme, calcineurin. Inhibition of this phosphatase activity has a number of effects<sup>540</sup>. It prevents the intracellular signalling which would normally occur in response to TCR stimulation and result in T cell activation and proliferation<sup>540</sup>. Notably, dephosphorylation of the nuclear factor of activated T cells (NFAT) is inhibited thereby preventing the translocation of NFAT to the nucleus, binding to its recognition sequence in the IL2 promotor and subsequent promotion of IL2 gene transcription<sup>540,541</sup>. Similarly, ciclosporin also inhibits the transcription of a number of other genes, including c-myc, cytokines such as IL-3, IL-4, IL-5 and IFN $\gamma$  and cytokine receptors such as the IL2 receptor. Conversely, ciclosporin upregulates transcription of TGF- $\beta$ 1 which may contribute to both the immunosuppressive properties of ciclosporin and fibrogenesis seen with long term ciclosporin use<sup>542</sup>.

Given these actions and the downstream pathways modulated by ciclosporin, it is not readily apparent how ciclosporin impacts on Shh gene expression and what the significance of this upregulation might be. That ciclosporin can act on targets other than calcineurin and that this may be responsible for some of its side effects including tumour formation and nephrotoxicity has been proposed previously<sup>543</sup>.

Another possibility is that ciclosporin is having a cytotoxic effect on the tubular cells and, indeed, one of the significant side effects of ciclosporin is nephrotoxicity<sup>544</sup>. Thus, the upregulated Shh expression may represent a response to a cytotoxic effect. The data presented here, however, would not lend support to frank cytotoxicity at the concentrations of ciclosporin employed. Ciclosporin is used here at concentrations below those considered to be cytotoxic in other studies<sup>501-503,505</sup>. Indeed, the results from the MTT assay would also lend support to the absence of frank cytotoxicity. It is entirely possible, however, that ciclosporin is having a damaging effect that is not readily detected by the MTT assay. The assay relies on the conversion of a colourless substrate to a coloured compound, conversion being proportional to the number of metabolically active cells present. Although there were no macroscopic differences between the wells or frank changes detected using trypan blue exclusion, it may be

that small differences in cell viability may not be detected by the assay. Whilst the MTT assay gives a crude estimate of cell death, it does not give an indication of injured cells which may be more relevant in this system. Other methodologies may be more useful. For example, the lactate dehydrogenase assay (LDH) may be more appropriate<sup>545</sup>. Alternatively, it may be possible to assess cell damage by evaluating markers of function such as sodium-dependent glucose transport and cAMP production induced by parathyroid hormone<sup>530</sup>.

It is interesting to speculate why ciclosporin might upregulate Shh expression and what the consequences of this upregulation might be. As discussed earlier in Chapter 3, one of the possible roles for Shh is that it may be acting as a danger signal, an endogenous factor released from damaged tissues in response to stress and capable of activating the immune system<sup>327,477</sup>. Like the proposed danger signals, the heat shock proteins, Shh is upregulated at sites of epithelial cell damage<sup>149</sup> and has the ability to activate cells of the peripheral immune system<sup>20,21</sup>. It is of particular interest then that ciclosporin has been shown to upregulate the expression and phosphorylation of the heat shock protein, HSP27, in the monkey renal proximal tubular cell line BSC-1<sup>543</sup> and, furthermore, that ciclosporin is reported to potentiate the synthesis of heat shock proteins induced by a heat shock<sup>546</sup>.

In this chapter, attempts were made to investigate whether the upregulation in *Shh* mRNA expression seen in ACHN cells was accompanied by release of Shh from the cells. This relied on the use of the murine Shh ELISA and Western blotting techniques to see whether loss of tissue Shh expression occurred in response to treatment and whether this was associated with the appearance of Shh protein in the supernatant. Attempts to delineate this were largely unsuccessful. Shh expression did not appear to vary greatly in the treated versus the untreated cells, and Shh was not detected using these techniques in the supernatants. Thus, these results may provide preliminary evidence to suggest that Shh is not released from the cells in response to ciclosporin treatment. With the availability of more robust Shh reagents, this could be further explored. For example, it is not entirely surprising that human Shh was not detected by the murine Shh ELISA, given the low reported cross-reactivity of the

ELISA for human Shh. Furthermore, determining Shh release into the supernatant by using the supernatants in Western blot experiments is not entirely satisfactory, not least because 100µg supernatant protein taken from the concentrated supernatant may only contain a very small quantity of Shh protein. Instead, the procedure could be further optimised using immunoprecipitation of the Shh from the supernatant prior to blotting. This involves the removal of Shh from the supernatant using an anti-Shh antibody. When mixed with Protein A/G resin, the antibody associates with the resin so that, with boiling, the antibody-protein complex dissociates and the antibody can be precipitated out with the resin, leaving the Shh protein in solution. This can then be used in a subsequent Western blotting experiment to detect for Shh. Ideally, the antibody used to probe for Shh in the blotting is a different antibody to that used previously for the immunoprecipitation. Attempts were made to perform this technique using combinations of the goat polyclonal antibody and the mouse monoclonal antibody, 5E1. However, 5E1, although able to detect rShh used as a positive control, failed to detect tissue-derived Shh. Similarly, attempts to use the goat polyclonal antibody failed to detect Shh in the supernatant although immunoprecipitation with rShh could be readily detected.



#### 4.7.2 Epithelial damage and Shh release

In the second part of this chapter, the effects of damaging agents on Shh release from REC cultures in 2 models of cell injury have been explored. Hydrogen peroxide and UVB have both been used previously to induce oxidative cell damage and apoptosis respectively in cell culture systems<sup>517-523,525-527,547</sup>. The results from this work would suggest that, in both forms of damage, however, Shh was not released from the cells. This could reflect 2 possibilities. Firstly, it may be that Shh is not released from these cells in response to damage. Indeed, it is tempting to speculate that such release occurs since Shh expression is upregulated at sites of epithelial damage<sup>149</sup> and numerous peripheral targets for Shh have been identified<sup>65,102,149</sup>. However, the results from this section of work would not support this occurrence. What is evident however is that protein expression is upregulated at sites of damage<sup>149</sup>. It has yet to be shown whether this results in the release of Shh. A further possible reason for the apparent lack of Shh release is that it failed to be detected by the systems employed for Shh detection as outlined earlier in this discussion. Indeed, the only method currently commercially available for detection of Shh release is the murine Shh ELISA used in this work. It is perhaps of interest that this ELISA has only been validated against recombinant Shh and Shh derived from cell lines overexpressing the cDNA vector. It has not been validated against tissue-derived Shh. It is entirely possible that the tissue-derived Shh is of a form not recognised by the ELISA. Indeed attempts to run through supernatants derived from centrifuged protein cell lysates of normal murine kidney were not detected by the ELISA suggesting that either cell-derived Shh is not detected by the ELISA; that the lysis solution interfered with the detection of Shh by the ELISA or that the amount of Shh present in the kidney was below the detection of the ELISA. Regarding this latter point, immunohistochemistry has been used by a number of groups to detect Hh proteins in a wide variety of cell systems. Although the same antibodies are often used in different studies, the results can vary dramatically with regards to the proposed expression reflecting the concentrations of antibodies used by different groups of workers. That Shh was detected in Chapter 3 using an antibody solution diluted to no more than 1:40 suggests that the level of expression may be quite low. This dilution was chosen to

be in keeping with previous studies from our group<sup>149</sup> and those of other authors<sup>160</sup>. However, in other studies of Shh expression, dilutions of antibody in the order of 1:250 have been used with good effect<sup>516</sup>. That renal epithelial Shh was only detected at 1:40 and not lower concentrations of antibody suggests that only very low levels of Shh protein may be expressed, perhaps too low to be detected by the ELISA.

Other possibilities also exist. For example, Shh may be released from tissue in a form which precludes detection by the Shh ELISA. Alternatively, it may be released in association with other known binding proteins such as the Shh receptor Ptc, Hip or dispatched<sup>37</sup>. Indeed Shh binds to Ptc at a region significantly overlapping with the binding region for the Shh antibody and, therefore, could readily mask Shh from detection. Furthermore, factors affecting ligand stability may also modulate the ability of soluble Shh to be detected by an ELISA. Indeed, recent reports by Bornemann et al<sup>548</sup> would suggest that the stability of Shh ligand may be significantly altered by heparan sulphate proteoglycans (HSPGs), thus potentially affecting the ability of the ELISA to detect Shh in the culture medium.

In light of the few systems available for detecting Shh release, it may be some time before this situation can be further clarified. A bioassay consisting of Shh-responsive cells has been developed by Beachy et al<sup>64</sup> and used previously to investigate activation of the Hh pathway. A clonal NIH3T3 cell line, Shh-LIGHT2, stably incorporating Gli-dependent firefly luciferase and constitutive *Renilla* luciferase reporters forms the basis of this assay, such that active Shh signalling induces luciferase activity. Another alternative would be to use another cell-based bioassay for Shh, the differentiation of C3H10T1/2 cells<sup>549</sup> whose read-out is alkaline phosphatase activity, as has been used elsewhere in the literature<sup>550</sup>. Use of such assays for the further evaluation of cell culture supernatants would aid greatly in determining whether Shh is released from epithelium in response to damage.

### 4.7.3 Summary

This chapter has explored factors that might influence the expression of Shh by renal epithelium. Two cell culture systems have been employed, the renal adenocarcinoma cell line, ACHN, and primary cultures of murine renal epithelial cells. These systems have been used to investigate the effect of the immunosuppressive agents, ciclosporin and dexamethasone, on Shh expression. In addition, and in light of previous reports in the literature of relevance to this work, the effects of damage on Shh expression in renal epithelial cells has been investigated. The results suggest that, in the ACHN cultures, Shh expression is upregulated in response to ciclosporin. Conversely, little effect is observed in response to dexamethasone and, in primary murine epithelial cells, Shh expression is not grossly modulated in response to either ciclosporin or dexamethasone. Release of Shh from epithelium does not appear to occur in response to either the immunosuppressive agents or, indeed, in response to 2 commonly used damaging modalities. However, the experiments outlined in this chapter have been largely hampered by the notably few reagents available for Shh detection and investigation of the Shh pathway. Thus, until further reagents for the Shh pathway are available, determining the importance and relevance of the findings presented here will remain difficult.

## **Chapter 5**

### **The Effect of Sonic Hedgehog on Human Macrophage Effector Function**

## 5.1 Introduction

Recent evidence has suggested that Sonic hedgehog (Shh) signalling may play a role in peripheral immunity<sup>20,21</sup>. Murine and human CD4+ T cells express Shh and its receptor, *Ptc*, and activated CD4+ T cells may respond to Shh through enhanced proliferation and modulation of cytokine production<sup>20,21</sup>. Much less, however, is known regarding a possible role for Shh signalling in the macrophage. *Ptc*, *Smo* and *Gli1-3* expression have been reported in primitive human pluripotent haematopoietic stem cells using RT-PCR<sup>19</sup> and the same group observed *Ptc* and *Smo* expression in mature myeloid (CD33+) cells<sup>19</sup>. More recently, murine alveolar macrophages have also been shown to express *Ptc*<sup>149</sup>.

The aim of these experiments was to determine whether adult human macrophages express components of the Shh signalling pathway and, furthermore, whether they are capable of responding to a Shh signal.

## 5.2 Shh signalling in human macrophages

### 5.2.1 Shh signalling pathway components are expressed in human macrophages

As described in Chapter 1, Shh signalling occurs through the interaction of Shh with its receptor Ptc<sup>40,49,551</sup>. In the absence of Shh binding, Ptc blocks the intrinsic activity of a further signal transduction protein, Smoothened (Smo). Shh binding relieves this blockade allowing pathway activation to occur<sup>46,47,552,553</sup> and, subsequently, members of the Gli family of zinc finger transcription factors are activated<sup>3,78,554</sup>.

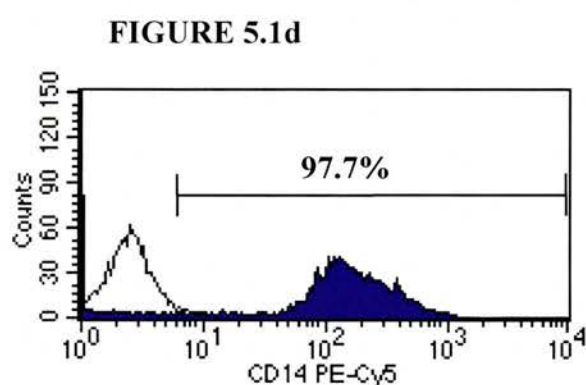
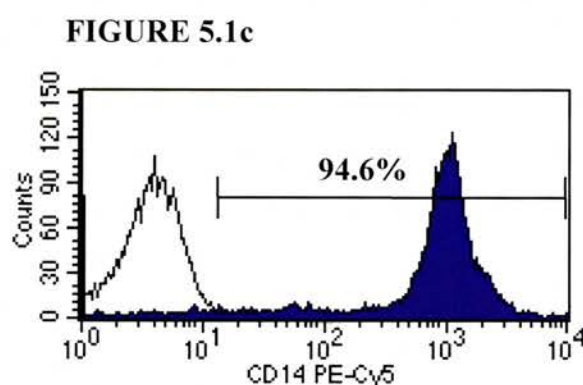
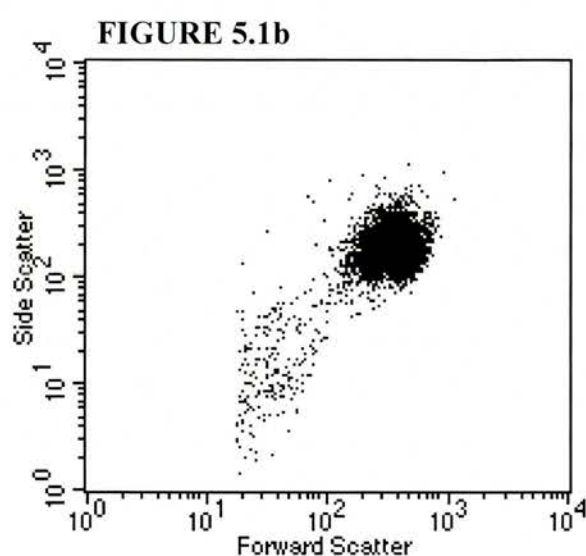
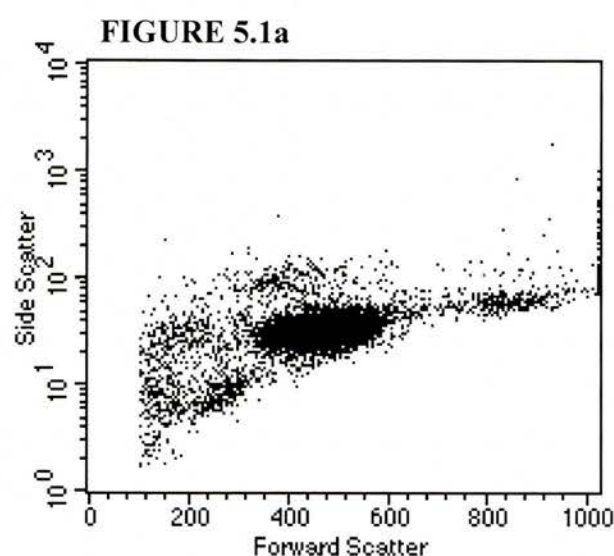
In these initial experiments, the expression of Shh, Ptc, Smo and members of the Gli family were evaluated.

Peripheral blood mononuclear cells (PBMCs) were prepared from normal human donor Buffy coats by centrifugation over Histopaque 1077 (Sigma) as described previously<sup>20,420,421</sup> and outlined in Chapter 2. Monocytes were isolated from the PBMCs by positive selection using CD14-labelled magnetic beads (Miltenyi Biotech, Bisley, Surrey UK). Following this isolation, purities of >93% were consistently obtained as determined by flow cytometry using a Phycoerythrin (PE) – Cy5 mouse anti-human CD14 antibody (Serotec, Kidlington, Oxford, UK) (Figure 5.1a and c). The isolated monocytes were matured into macrophages by plastic adherence for 6 days as described in Chapter 2. During this adherence step, the cells were washed and the medium replaced every 2 days such that, by the end of this time, the cells were consistently >96% CD14+ (Figure 5.1b and d). RNA was extracted from the macrophages using Trizol extraction and RT-PCR performed to determine the expression of the Shh signalling pathway components *Shh*, *Ptc*, *Smo*, *Gli 1* and *Gli 2*. As shown in Figure 5.2a, macrophages express the signalling pathway components *Ptc*, *Smo*, *Gli-1* and *Gli-2*. However, although specific transcripts for *Shh* have been identified in CD4+ T cells<sup>20,21</sup> and were present in the PBMCs that served as a positive control, transcripts for *Shh* mRNA were not identified in macrophages from any of the 5 donors studied.



To determine protein expression, immunohistochemistry was performed using macrophages plated onto glass coverslips as described in Chapter 2. Polyclonal antibodies to Shh, Ptc, Smo and Gli 1 (Santa Cruz Biotechnology, Insight Biotechnology Ltd, Wembley, UK) were used to evaluate the macrophage expression of these proteins. It was not possible to determine the expression of Gli 2 in these experiments, as antibodies to Gli 2 are not currently commercially available. To check specificity, the same antibodies were pre-incubated with the appropriate specific blocking peptides (Santa Cruz Biotechnology). The blocking peptides are designed to bind to the epitope of the antibody responsible for binding to the protein of interest and thus, when used in excess, should not allow binding of the antibody to the specific protein to occur. In accordance with the manufacturer's instructions, the antibodies were each pre-incubated with a five-fold excess (by weight) of blocking peptide and then applied to the cells in experiments performed in parallel with experiments in which primary antibodies were applied to the cells. Cells treated in this way served as negative controls to the experiments.

As demonstrated in Figure 5.2b, Ptc was expressed predominantly on the macrophage cell surface and this staining could be blocked using the specific Ptc-blocking peptide. Conversely, although Shh, Smo and Gli 1 appeared to be expressed in the macrophage, this staining could not be abrogated using the appropriate specific blocking peptides suggesting that the positive staining may be non-specific in nature.

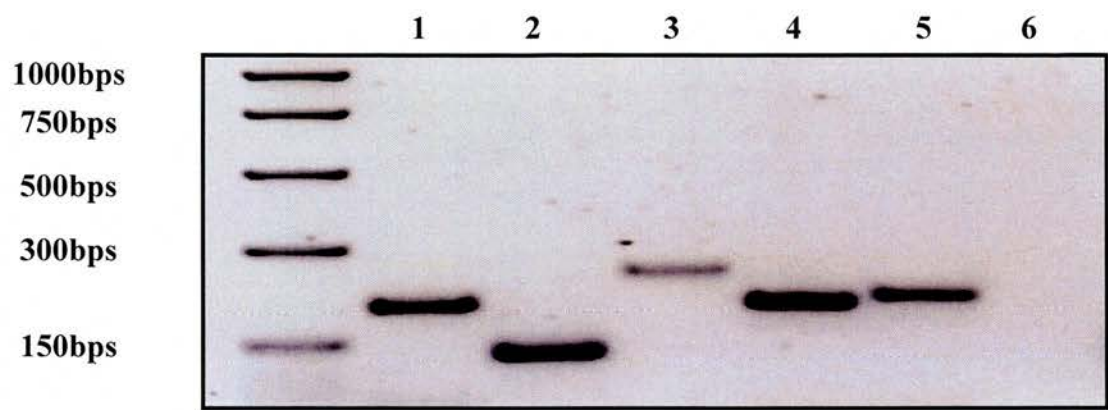


**FIGURE 5.1**

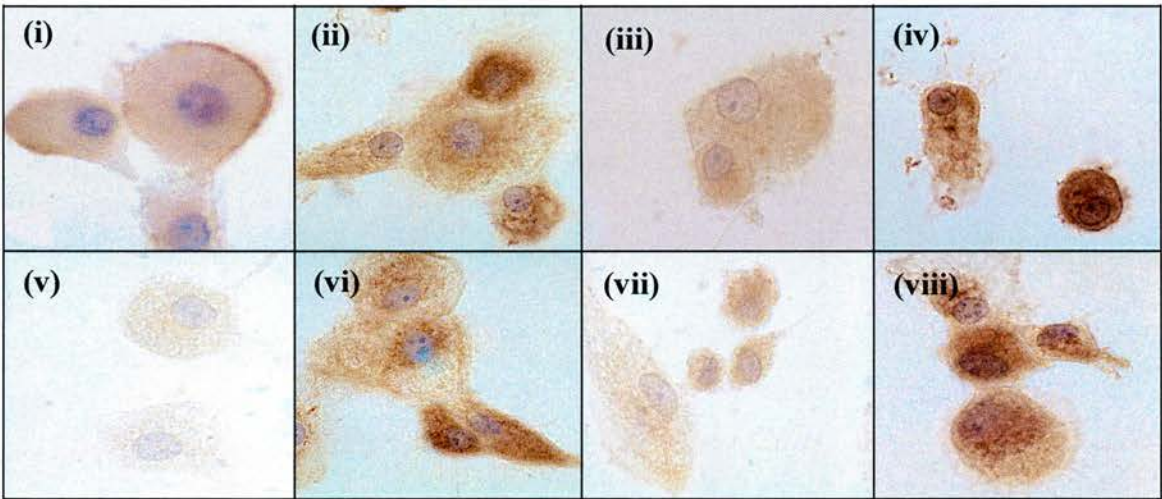
**Representative flow cytometry data demonstrating purities of macrophages used for macrophage experiments.**

Following centrifugation over Histopaque, monocytes were isolated from PBMCs by magnetic bead separation using CD14 positive selection beads. The monocytes were adhered for a period of 6 days. Representative flow cytometry scatter plots for unstained monocytes (Figure 5.1a) and Day 6 macrophages (Figure 5.1b) are shown. Purities of monocytes (Figure 5.1c) and macrophages (Figure 5.1d) were confirmed using a PE-Cy5 mouse anti-human antibody (shaded histograms) and percentages represent the purity of the cells compared to the mouse IgG2a isotype control (unshaded histograms) in each case.

**FIGURE 5.2a**



**FIGURE 5.2b**



**Mag x 1000**

**FIGURE 5.2**

**The Shh signalling components *Ptc*, *Smo*, *Gli 1* and *Gli 2* are expressed in human PBMC-derived macrophages.**

**5.2a** RT-PCR was used to demonstrate the expression of *Ptc* (Lane 1, 200bps), *Smo* (Lane 2, 140bps), *Gli 1* (Lane 3, 244bps) and *Gli 2* (Lane 4, 200bps). *Shh* was expressed in the PBMCs that served as a positive control (Lane 5, 211bps) but not in macrophages (Lane 6).

**5.2b** Immunohistochemistry was used to demonstrate protein expression of *Ptc* (i), *Smo* (ii), *Gli 1* (iii) and *Shh* (iv) in human macrophages. Specificity of staining was tested using the appropriate specific blocking peptides for *Ptc* (v), *Smo* (vi), *Gli 1* (vii) and *Shh* (viii). Staining for *Ptc*, but not *Smo*, *Gli1* and *Shh*, could be blocked using the appropriate blocking peptide.

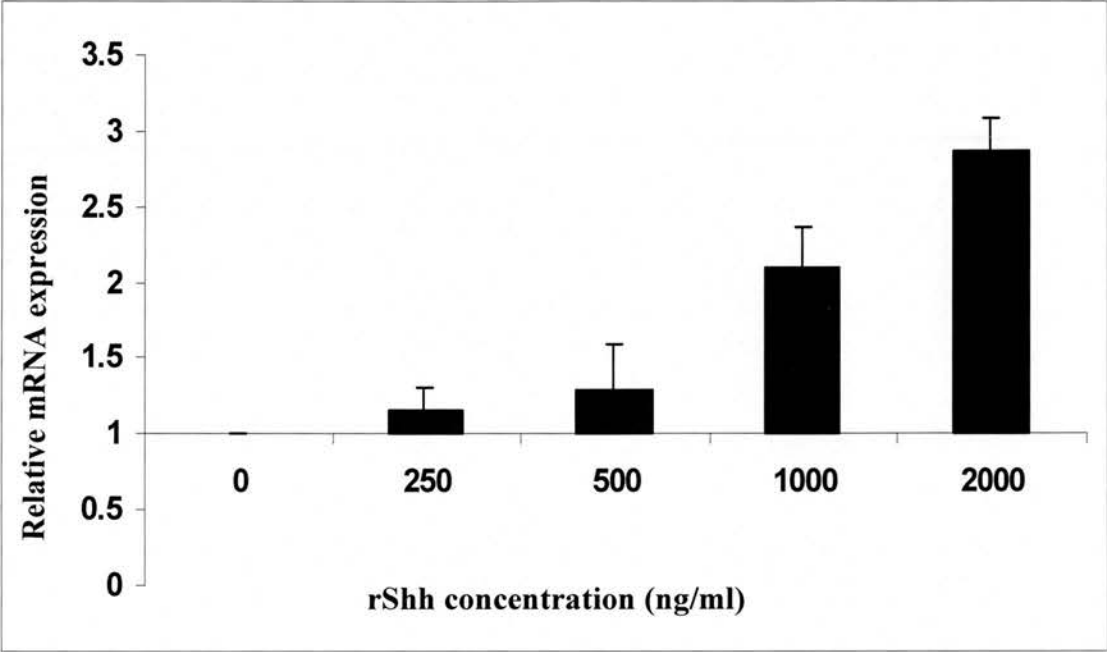
### 5.2.2 *Ptc* expression is upregulated in response to recombinant Shh (rShh)

Given that human macrophages express *Ptc* and other components of the Shh signalling pathway, at least at the mRNA level, the aim of these experiments was to determine whether active Shh signalling occurs in these cells. In addition to its role as a receptor for the Shh signal, *Ptc* also acts as a transcriptional target of Shh<sup>16,20,21,32,44,99,100,102</sup>. Thus, upregulation of *Ptc* expression is widely considered to reflect active signalling through the Shh pathway. The aim of these experiments was to determine whether *Ptc* upregulation occurs in macrophages in response to Shh stimulation.

For these and subsequent experiments, a recombinant mouse Sonic hedgehog peptide (rShh) (R&D Systems, Abingdon, UK) was used to stimulate the macrophages. This was the only peptide commercially available at the start of my PhD. It differs from human Shh in only one amino acid and has been used elsewhere for human studies<sup>20,65,510</sup>. Midway through my PhD, a recombinant human peptide (Peprotech EC Ltd, London, UK) became available. This was shown to induce the same effector function changes in the macrophages as those which will be discussed in this chapter. However, to maintain consistency, the recombinant murine form of the peptide was used for all experiments described herein.

PBMC-derived macrophages were stimulated with rShh in the dose range 250ng/ml – 2000ng/ml, as described in Chapter 2. This dose range has been shown previously to modulate the effector of human and murine CD4+ T cells<sup>20,21</sup>. In keeping with previous *in vitro* studies<sup>20</sup>, following a 24 hour incubation with rShh, RNA was extracted from untreated (control) cells and rShh-treated cells. *Ptc* expression was evaluated using real-time PCR using 18S as an internal control. Each sample was run on a real-time plate in duplicate and the experiment was performed using RNA from 5 different donors.

As demonstrated in Figure 5.3, *Ptc* expression was upregulated in a dose-dependent manner in response to rShh in the 5 donors studied.



**FIGURE 5.3**

***Ptc* expression is upregulated in macrophages in response to rShh**

Real-time PCR was performed to determine the expression of *Ptc* mRNA in macrophages in response to rShh. Values are expressed as a ratio of the concentration of *Ptc* mRNA relative to untreated macrophages grown in medium alone. The untreated (0ng/ml rShh) macrophages are given a value of 1. Results represent experiments performed on 5 individual donors and means and standard deviations are shown.

Collectively, these results show that macrophages express components of the Shh signalling pathway and that, in response to rShh, *Ptc* mRNA expression is upregulated. Taken together, these results suggest that active signalling occurs through the Shh signalling pathway in response to rShh.



## 5.3 rShh modulates the effector function of macrophages

Although there have been previous reports describing the expression of Shh signalling pathway components in cells of myeloid lineage<sup>19,149</sup>, there have been no reports in the literature describing possible roles for activation of the signalling pathway in these cells. Therefore, the aim of this series of experiments was to determine whether activation of the Shh signalling pathway was capable of inducing a change in the effector function of macrophages.

### 5.3.1 rShh differentially modulates the Vascular Endothelial Growth Factor and Transforming Growth Factor- $\beta$ expression by macrophages

Although the role of Shh signalling in the macrophage has not been investigated to date, there has been evidence suggesting a link between the Shh signalling pathway and two recognised macrophage products, Vascular Endothelial Growth Factor (VEGF) and Transforming Growth Factor Beta (TGF- $\beta$ ).

As discussed in Chapter 1, VEGF is a potent endothelial cell mitogen, angiogenic factor, vasodilator and enhancer of microvascular permeability<sup>442,555-557</sup>. That Shh may be involved in VEGF signalling and angiogenesis is suggested by several recent reports in the literature<sup>65,102,103,163</sup>. In 2001, Pola et al demonstrated VEGF upregulation in myofibroblasts in response to Shh signalling and, *in vivo*, Shh signalling was shown to induce neovascularisation in hindlimbs damaged by ischaemia<sup>102</sup>. More recently, studies in the zebrafish embryo have supported these findings<sup>163</sup> with the injection of Shh mRNA inducing *VEGF* mRNA expression in this model. Furthermore, Kanda et al<sup>65</sup> demonstrated capillary morphogenesis in cultured endothelial cells in response to rShh.

The TGF- $\beta$  superfamily consists of a large number of growth factors all sharing conserved cysteine residues in their C-terminal region<sup>118</sup>. Several members of the TGF- $\beta$  superfamily are involved with and regulated by the Hh signalling pathway.

For example, the bone morphogenetic proteins (BMPs)<sup>4,119,427</sup> and, in *Drosophila*, the morphogen Decapentaplegic (Dpp) are both members of the TGF- $\beta$  superfamily and downstream targets of Hh signalling<sup>110</sup>. Furthermore the segment polarity gene *Wingless (wg)*<sup>437</sup> is a downstream target of Hh signalling<sup>437,438</sup> and interactions between this pathway and TGF- $\beta$  signalling have been described<sup>117,120</sup>.

The aim of these initial experiments was to determine whether Shh signalling modulates the production of TGF- $\beta$  and VEGF from macrophages.

Adherence-matured macrophages were stimulated with rShh (500-2000ng/ml) over the time course 3-72 hours and the supernatants harvested for VEGF and TGF- $\beta$ 1 analysis using ELISAs as described in Chapter 2. The TGF- $\beta$ 1 DuoSet ELISA (R&D Systems) allows for the quantification of both active and latent TGF- $\beta$ 1. In accordance with the manufacturer's instructions, to detect latent TGF- $\beta$ 1, each sample was acidified using 1N hydrochloric acid and then neutralised using 2.7N Sodium hydroxide/0.5M HEPES. These treated samples were then run in parallel with the untreated samples on a 96 well ELISA plate, thus allowing latent and activated TGF- $\beta$ 1 to be assayed for each sample. To circumvent the well-recognised concern that serum can contain significant amounts of latent TGF- $\beta$ 1<sup>558,559</sup>, macrophages were cultured for 6 days either in the presence (Iscoves Modified Dulbeccos medium supplemented with 5% fetal calf serum; IMDM-FCS, Gibco) or absence of serum (X-Vivo 10 serum-free medium, Cambrex BioScience, Wokingham, Berkshire, UK) prior to stimulation with rShh.

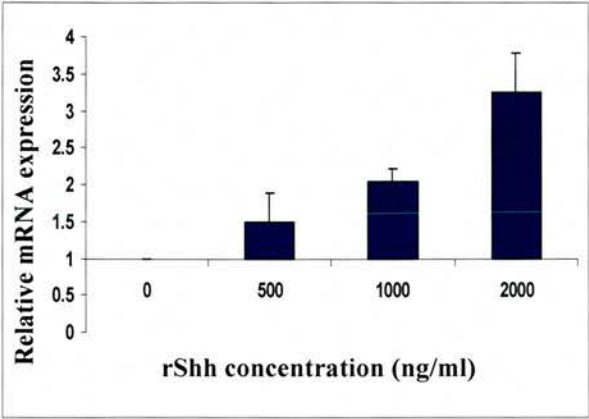
The results illustrated in Figures 5.4 and 5.5 reflect the response of macrophages derived from 5 individual donors to rShh. As shown in Figure 5.4, the addition of rShh induced a slight upregulation in the production of VEGF protein in macrophages cultured in IMDM-FCS which was, at the highest concentration of rShh used, at levels just above the level of sensitivity of the ELISA (15pg/ml). RNA extracted from these macrophages revealed a dose-dependent upregulation of *VEGF* mRNA expression. Conversely, in macrophages cultured in serum-free conditions, significant modulation by rShh of VEGF protein or mRNA was not observed in any

of the 5 donors studied (Figures 5.4c and d) irrespective of the time point studied (data for 24 hours illustrated in Figure 5.4).

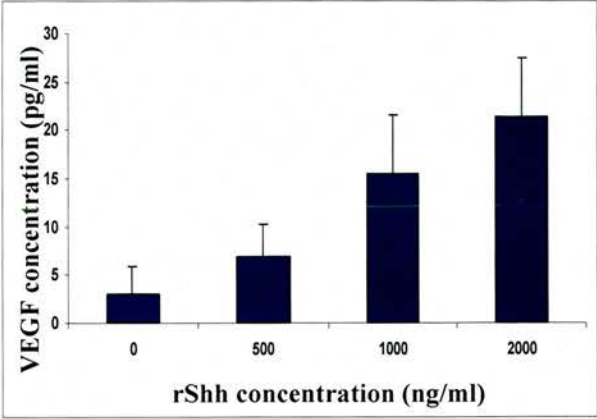
Figure 5.5 illustrates the effect of rShh on the TGF- $\beta$ 1 production by macrophages at 24 hours. As shown, neither latent nor active TGF- $\beta$  was modulated by rShh in any of the donors studied and this was observed irrespective of whether serum-containing or serum-free medium was used and irrespective of the time point studied (data for 24 hours illustrated in Figure 5.5).

Thus, the findings from this series of experiments would broadly support the previous observations by Pola et al<sup>102</sup> suggesting that rShh upregulates VEGF expression. However, in the present series of experiments, the upregulation of VEGF appeared to be dependent on the presence of serum in the culture conditions and an upregulation in VEGF expression was not observed under serum-free conditions. TGF- $\beta$  was not modulated by rShh in macrophages irrespective of the presence or absence of serum.

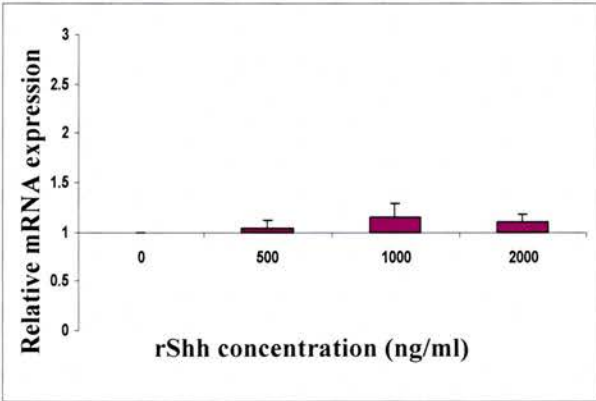
**FIGURE 5.4a**



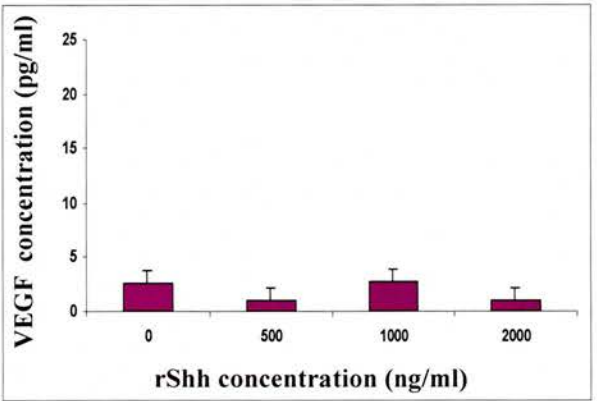
**FIGURE 5.4b**



**FIGURE 5.4c**



**FIGURE 5.4d**

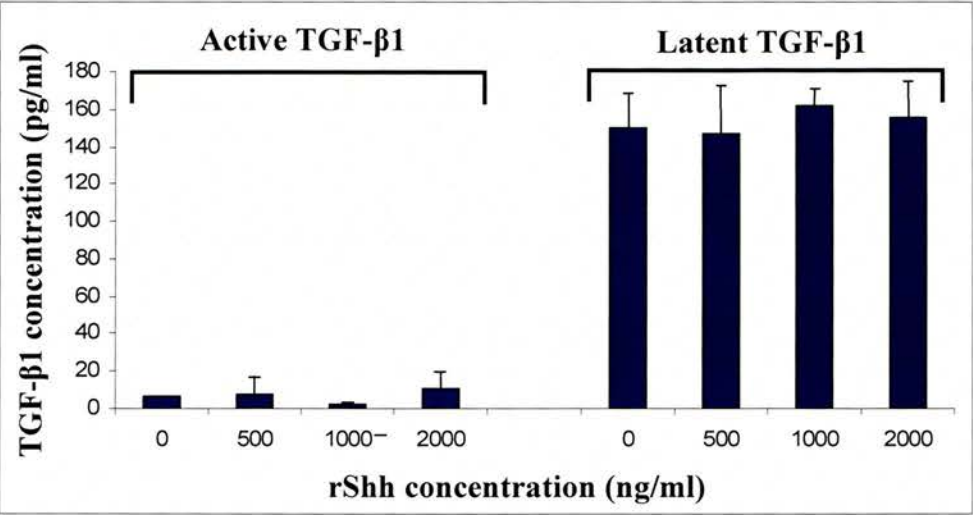


**FIGURE 5.4**

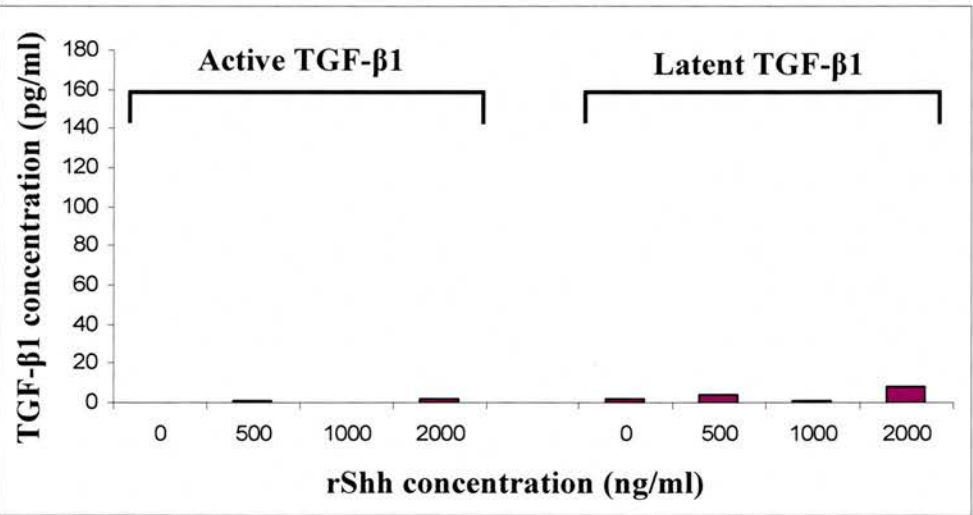
**rShh differentially modulates macrophage VEGF expression**

Macrophages were stimulated with rShh (500ng/ml-2000ng/ml) and supernatants and RNA harvested to determine VEGF protein and mRNA expression using ELISA and real-time PCR respectively. Results from 24 hour cultures with rShh are shown. Macrophages cultured in IMDM-FCS responded to rShh by upregulating VEGF mRNA (a) and protein (b) expression in a dose-dependent manner. Conversely, macrophages incubated in serum-free medium failed to demonstrate upregulated VEGF mRNA (c) or protein (d) expression. Results represent experiments performed on 5 individual donors and means and standard deviations are shown. The limit of detection for the VEGF ELISA is 15pg/ml.

**FIGURE 5.5a**



**FIGURE 5.5b**



**FIGURE 5.5**

**rShh fails to modulate TGF-β production by macrophages**

PBMC-derived macrophages were stimulated with rShh (500ng/ml-2000ng/ml) and supernatants harvested to determine TGF-β1 production using ELISA. Results from supernatants harvested after a 24 hour incubation with rShh are shown. Irrespective of whether FCS-containing medium (a) or X-Vivo 10 serum-free medium (b) was used to culture the macrophages, rShh failed to modulate either the active or latent TGF-β1 production. Results represent experiments performed on 5 individual donors and means and standard deviations are shown. The limit of detection for the TGF-β ELISA is 15pg/ml.

### **5.3.2 rShh differentially modulates the cytokine and chemokine expression by macrophages**

Given the upregulation of VEGF by rShh, this prompted the question as to whether rShh may also modulate the expression of other inflammatory cytokine and chemokines by macrophages. Recent evidence suggests that active Shh signalling may be important at sites of inflammation. Upregulated Shh expression has been reported at sites of epithelial inflammation in the lung, both in human cryptogenic fibrosing alveolitis and murine models of fibrotic lung disease<sup>149</sup> and, in these same sites, Ptc is expressed on the infiltrating mononuclear cells and alveolar macrophages<sup>149</sup>. Furthermore, Shh has been reported to enhance the proliferation of CD4+ T cells<sup>20,21</sup>, an effect accompanied by a modulation in the cytokines produced by these cells<sup>20</sup>. Notably, IL-2 and IFN- $\gamma$  appear to be at least partly dependent on the Shh pathway as evidenced by some inhibition of this release when Shh signalling is blocked. Together, these results would suggest that Shh signalling may be important at sites of inflammation and the following experiments were performed to determine whether Shh plays a role in modulating the cytokine and chemokine effector function of macrophages.

Macrophages were prepared under serum-containing and serum-free conditions as in Section 5.3.1 and stimulated with rShh (500-2000ng/ml) for 1-72 hours. Supernatants were harvested for cytokine and chemokine analysis using the Inflammatory and Chemokine Cytometric Bead Arrays (CBAs) (BD Biosciences, Cowley, Oxford, UK) as outlined in Chapter 2. Using the Inflammatory and Chemokine CBAs the following cytokines and chemokines can be detected: Interleukins -1 beta (IL-1 $\beta$ ), -12p70, -6 and -8, Tumour necrosis factor alpha (TNF $\alpha$ ), Monocyte chemoattractant protein - 1 (MCP-1), Interferon gamma-inducible protein (IP-10), Monokine induced by interferon gamma (MIG) and Regulated on activation, normal T cell expressed and secreted (RANTES).



Figures 5.6 and 5.7 demonstrate the effect of rShh on cytokine and chemokine release at 24 hours in the presence of serum. For the purposes of statistical analysis, treated macrophages were compared to untreated controls using a two-tailed t-test for paired samples. A p value of <0.05 was considered significant (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

rShh upregulated the production of IL-6, IL-8, IP-10, MCP-1, MIG and RANTES in a dose-dependent manner when macrophages were cultured in the presence of serum, this reaching significance at all concentrations of rShh used (Figure 5.6 (a-f)).

Conversely, under the same conditions, rShh failed to modulate the production of IL-1 $\beta$ , IL-12p70 (Figure 5.7 a and b). TNF $\alpha$  and IL-10 were upregulated in only some donors (8 of 20, and 7 of 20 respectively) but following statistical analysis, this upregulation was not statistically significant (Figure 5.7 c and d).

When macrophages were cultured in the absence of serum (Figure 5.8), rShh also induced an upregulation in IL-6, IL-8, MCP-1, IP-10, MIG and RANTES, albeit that the magnitude of the response was less marked. IL-1 $\beta$ , IL-12p70, TNF $\alpha$  and IL-10 failed to be upregulated by rShh under serum-free conditions (Figure 5.9) in keeping with the results obtained when cells were cultured in the presence of serum.

Furthermore, rShh failed to modulate these cytokines irrespective of the time point studied (1-72 hours, data not shown). This is important given that temporal differences in expression profiles in response to different stimuli have been reported<sup>420,560,561</sup>. For example, peaks in TNF $\alpha$  production classically appear early (4-8 hours) and cyclical peaks in protein expression have also been reported<sup>562,563</sup>.

Thus, the results from this section suggest that rShh differentially modulates the cytokine and chemokine production from human macrophages. Where rShh upregulates the production of cytokines and chemokines, this effect is markedly enhanced in the presence of serum.

FIGURE 5.6a

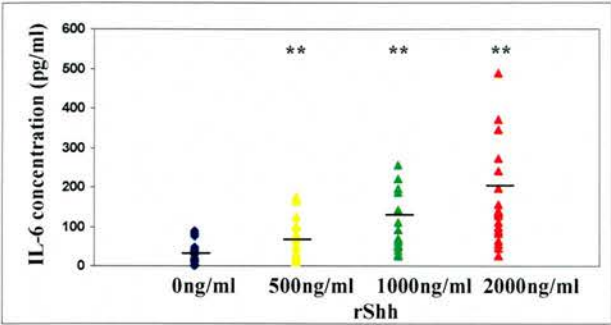


FIGURE 5.6b

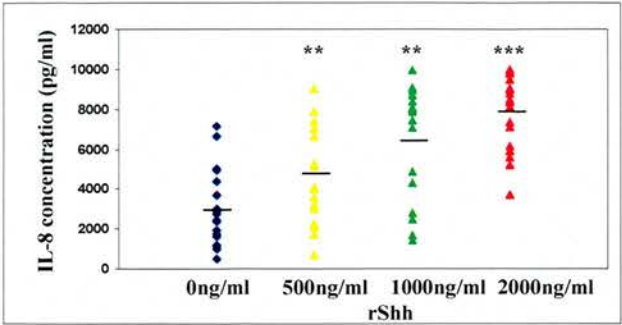


FIGURE 5.6c

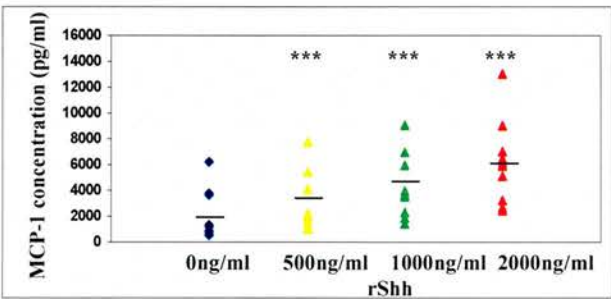


FIGURE 5.6d

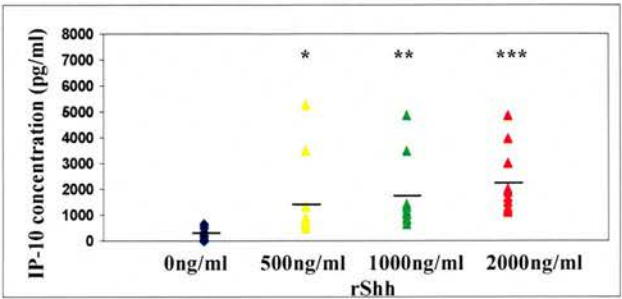


FIGURE 5.6e

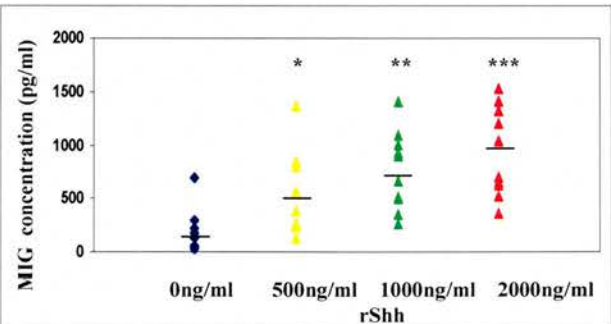


FIGURE 5.6f

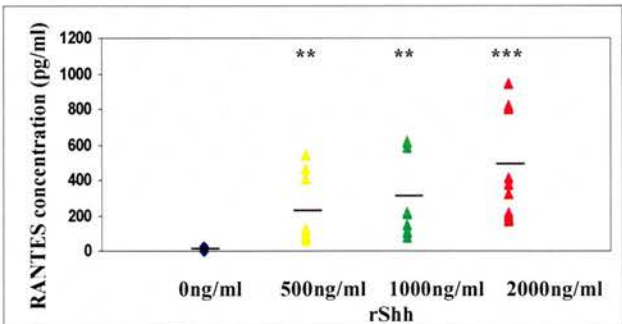
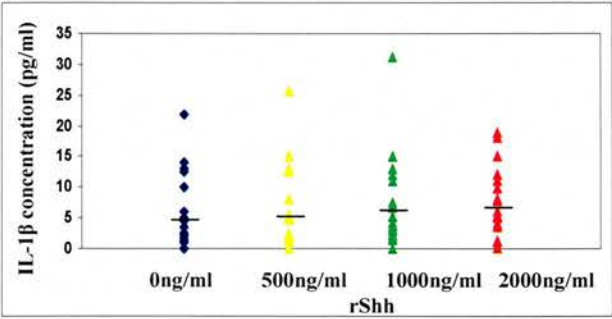


FIGURE 5.6

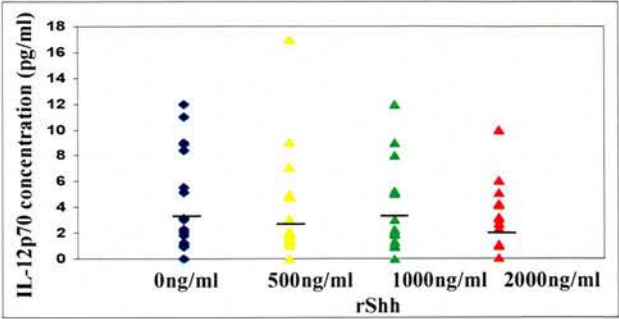
**rShh upregulates the production of IL-6, IL-8, MCP-1, IP-10, MIG and RANTES by macrophages cultured in the presence of serum.**

Macrophages were cultured in the presence of rShh (500-2000ng/ml) for 1-72 hours. Supernatants were harvested and analysed by CBA. Graphs represent cytokine production from 20 donors and chemokine production from 10 donors and results from the 24 hour time point are illustrated. rShh upregulated the production of IL-6 (a), IL-8 (b), MCP-1 (c), IP-10 (d), MIG (e) and RANTES (f). For the purposes of statistical analysis, treated macrophages were compared to untreated controls using a two-tailed paired t-test. A p value of <0.05 was considered significant (\* p<0.05, \*\* p<0.01, \*\*\* p<0.001). The means and individual data points are shown.

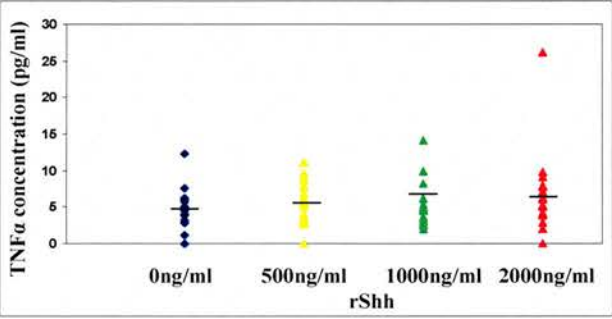
**FIGURE 5.7a**



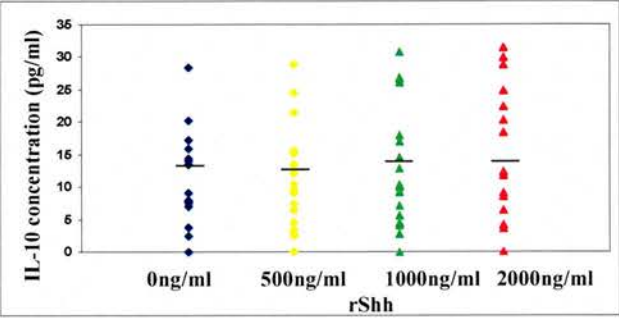
**FIGURE 5.7b**



**FIGURE 5.7c**



**FIGURE 5.7d**

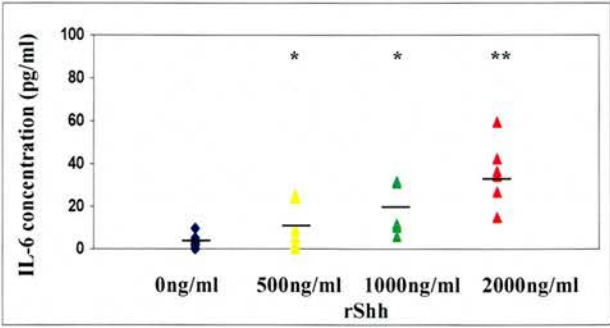


**FIGURE 5.7**

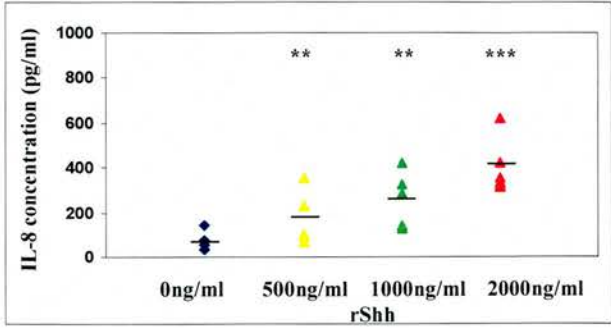
**rShh fails to modulate the production of IL-1 $\beta$ , IL-12p70, TNF $\alpha$  or IL-10 by macrophages cultured in the presence of serum**

Macrophages were cultured in the presence of rShh (500-2000ng/ml) for 1-72 hours. Supernatants were harvested and analysed by CBA. Graphs represent cytokine production from 20 donors and results from the 24 hour time point are illustrated. rShh failed to modulate IL-1 $\beta$  (a), IL-12p70 (b), TNF $\alpha$ (c) and IL-10 (d) production by macrophages. For the purposes of statistical analysis, treated macrophages were compared to untreated controls using a two-tailed paired t-test. The means and individual data points are shown.

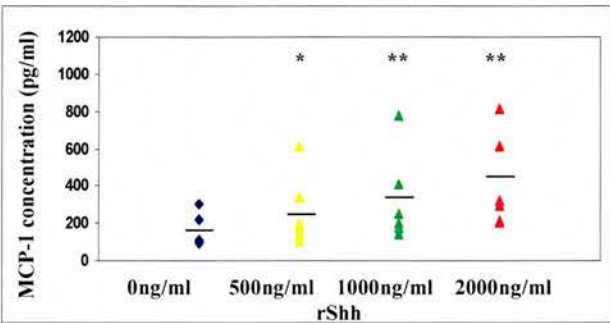
**FIGURE 5.8a**



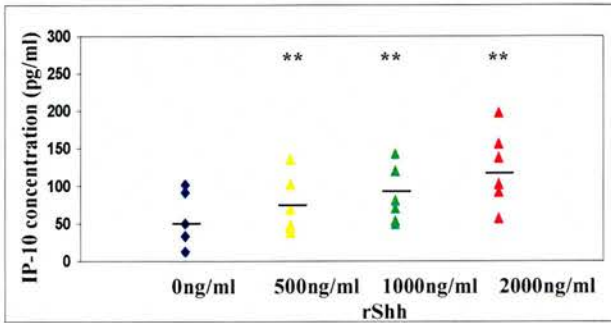
**FIGURE 5.8b**



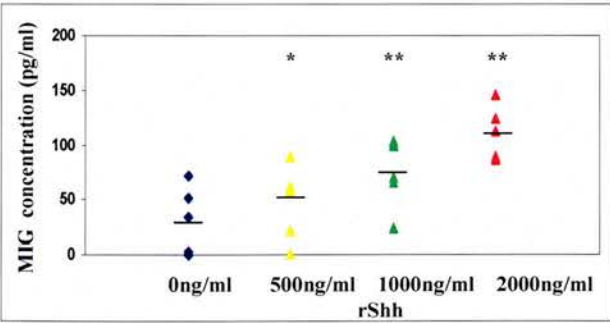
**FIGURE 5.8c**



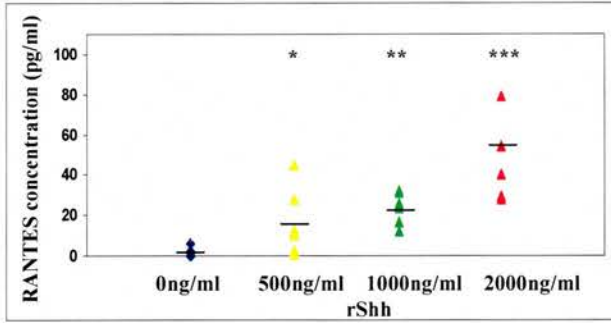
**FIGURE 5.8d**



**FIGURE 5.8e**



**FIGURE 5.8f**



**FIGURE 5.8**

**rShh upregulates the production of IL-6, IL-8, MCP-1, IP-10, MIG and RANTES by macrophages cultured in the absence of serum.**

Macrophages were cultured in the presence of rShh (500-2000ng/ml) for 1-72 hours. Supernatants were harvested and analysed by CBA. Graphs represent cytokine and chemokine production from 6 donors and results from the 24 hour time point are illustrated. rShh upregulated the production of IL-6 (a), IL-8 (b), MCP-1 (c), IP-10 (d), MIG (e) and RANTES (f). For the purposes of statistical analysis, treated macrophages were compared to untreated controls using a two-tailed paired t-test. A p value of <0.05 was considered significant (\* p<0.05, \*\* p<0.01, \*\*\* p<0.001). The means and individual data points are shown.



FIGURE 5.9a

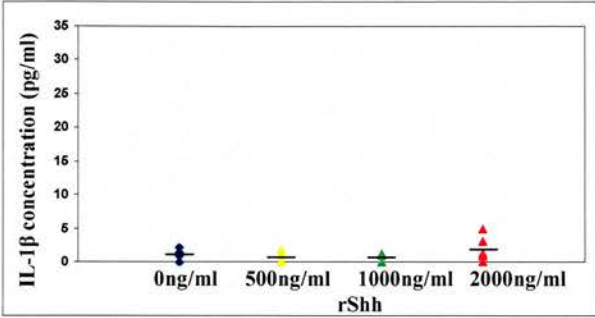


FIGURE 5.9b

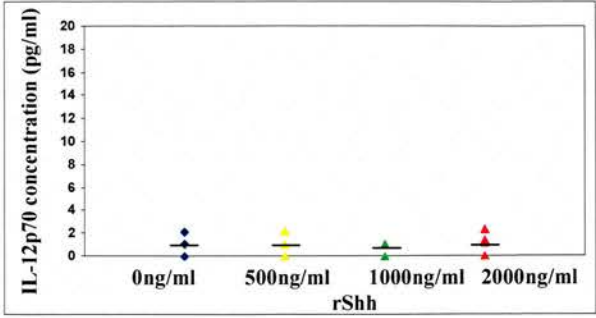


FIGURE 5.9c

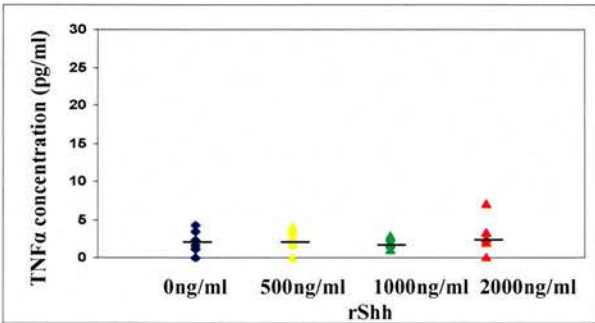


FIGURE 5.9d

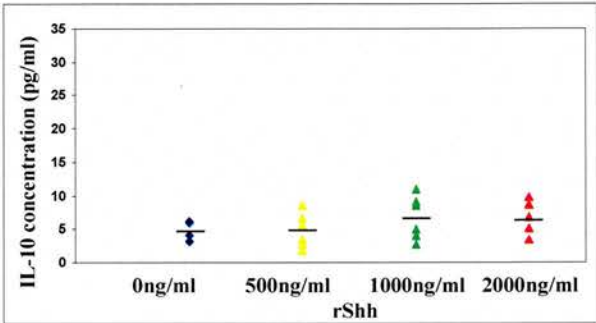


FIGURE 5.9

**rShh fails to modulate the production of IL-1 $\beta$ , IL-12p70, TNF $\alpha$  or IL-10 by macrophages cultured in the absence of serum.**

Macrophages were cultured in the presence of rShh (500-2000ng/ml) for 1-72 hours. Supernatants were harvested and analysed by CBA. Graphs represent cytokine production from 6 donors and results from the 24 hour time point are illustrated. rShh failed to modulate IL-1 $\beta$  (a), IL-12p70 (b), TNF $\alpha$ (c) and IL-10 (d) production by macrophages. For the purposes of statistical analysis, treated macrophages were compared to untreated controls using a two-tailed paired t-test. The means and individual data points are shown.

## 5.4 Inhibiting Shh signalling using cyclopamine differentially abrogates the macrophage response to rShh

From the experiments above, it would appear that macrophages express the receptor for Shh ligand, Ptc; that *Ptc* mRNA is upregulated in response to stimulation with Shh ligand and that the effector function of macrophages may be differentially modulated by Shh.

The purpose of this series of experiments was to determine whether the effect of Shh on macrophages could be abrogated by specifically inhibiting active Shh signalling. As described in Chapter 1, in the absence of Hh binding, Ptc exerts a repressive effect on the key transducer of the Shh signal, Smoothed (Smo)<sup>46,47,101,553</sup> and this repression is lifted with ligand binding.

Although Ptc is not required for signal transduction, Smo activation is required for signalling<sup>64</sup>. Thus, to specifically inhibit the Shh pathway, the *Veratrum* alkaloid and teratogen, cyclopamine, was used. This has been shown to specifically inhibit the cellular response to Shh<sup>17,64,69</sup> through an interaction with activated Smo<sup>60,62,64,70</sup> and inhibits Shh signalling across a wide concentration range (20nM - 20μM)<sup>17,64,65,68</sup>.

In an initial set of experiments, macrophages were pre-incubated in the presence of 5-20μM cyclopamine for 45 minutes. This concentration range was in keeping with that previously reported for *in vitro* experimentation in the literature<sup>64,65,68</sup>. rShh was then added to the culture medium giving a final rShh concentration range of 500-2000ng/ml. The cells were incubated under these conditions for 24 hours and the supernatants then harvested for cytokine and chemokine analysis by CBA. In the first 3 donors examined, cyclopamine inhibited 2000ng/ml rShh-induced IL-6 production at all three concentrations of cyclopamine used (5, 10 and 20μM) and thus, for all subsequent experiments, 10μM cyclopamine was used. At all concentrations of cyclopamine used and, in keeping with previous studies, there was no apparent



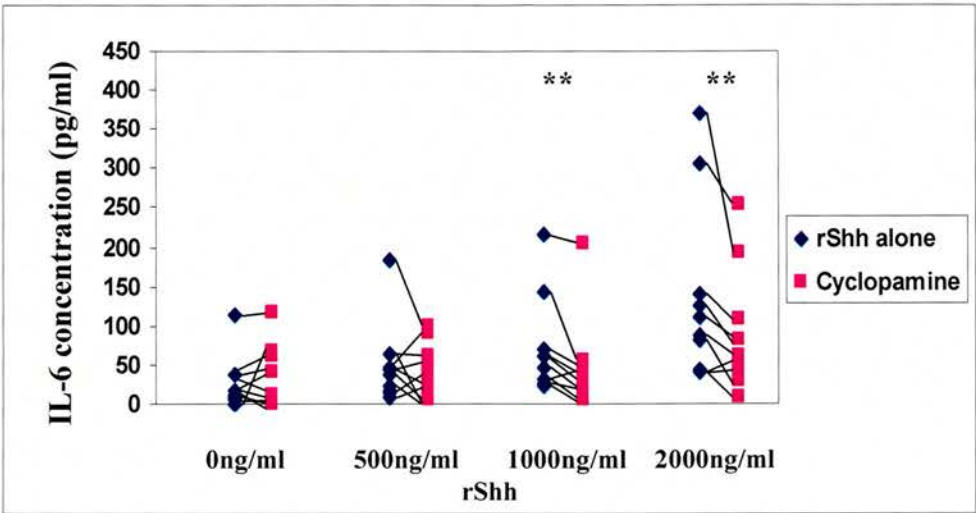
evidence of cell toxicity as determined using the MTT assay and trypan blue exclusion.

As shown in Figure 5.10, cyclopamine inhibited the rShh-mediated upregulation of IL-6 and MCP-1 in macrophages grown in the presence of serum. The IL-6 induced by 1000ng/ml and 2000ng/ml rShh was significantly inhibited by 10 $\mu$ M cyclopamine ( $p=0.013$  and  $p=0.027$  respectively). Similarly, the MCP-1 induced by 1000ng/ml and 2000ng/ml rShh was also inhibited ( $p=0.047$  and  $p=0.006$  for 1000ng/ml and 2000ng/ml rShh respectively). Neither IL-6 nor MCP-1 were inhibited significantly when 500ng/ml rShh was used ( $p=0.64$  and  $p=0.87$  respectively). IL-8 was inhibited in some donors (4 of 8). Conversely, the rShh-mediated upregulation of RANTES, MIG and IP-10 was not modulated by cyclopamine in any of the 8 donors studied (Figure 5.11).

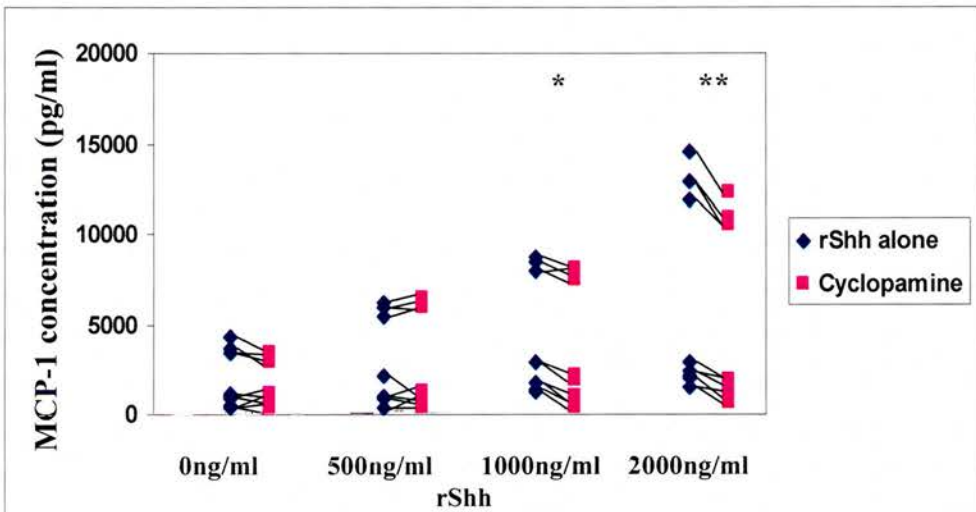
When the experiments were repeated in serum-free conditions, the effect of cyclopamine on IL-6 and MCP-1 production was much less marked, only reaching statistical significance at the highest concentration of rShh used (Figure 5.12). IL-8 was inhibited in 4 of 10 donors and, as in the presence of serum, RANTES, MIG and IP-10 were not modulated by cyclopamine (Figure 5.13).

Thus, the results from this series of experiments reveal that the specific inhibitor of the Shh signalling pathway, cyclopamine, differentially modulates the rShh-induced upregulation of cytokines and chemokines. This effect is most marked in those cell cultures containing serum.

**FIGURE 5.10a**



**FIGURE 5.10b**



**FIGURE 5.10**

**Cyclopamine inhibits the rShh-mediated upregulation of IL-6 and MCP-1 in macrophages cultured in the presence of serum**

Macrophages were pre-incubated with 10 $\mu$ M cyclopamine for 45 minutes. rShh was added to the medium to give a final concentration 500-2000ng/ml. As previously, supernatants were harvested for cytokine and chemokine analysis by CBA. Represented are the results from 10 individual donors. The rShh-mediated upregulation of IL-6 (Figure 5.10a) and MCP-1 (Figure 5.10b) was inhibited by cyclopamine at the higher concentrations of rShh used. For the purposes of statistical analysis, cyclopamine treated macrophages were compared to rShh-alone controls using a two-tailed paired t-test. A p value of <0.05 was considered significant (\* p<0.05, \*\* p<0.01, \*\*\* p<0.001). The means and individual data points are shown.

FIGURE 5.11a

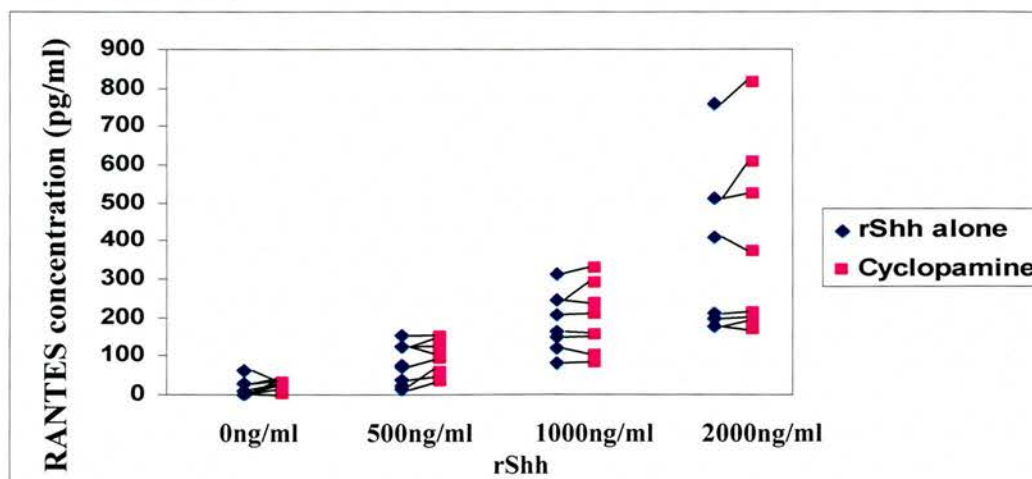


FIGURE 5.11b

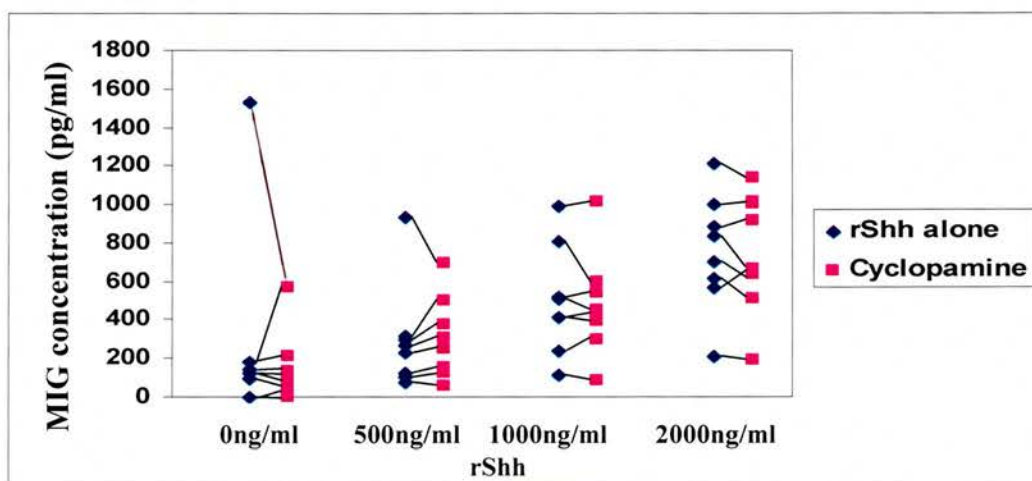


FIGURE 5.11c

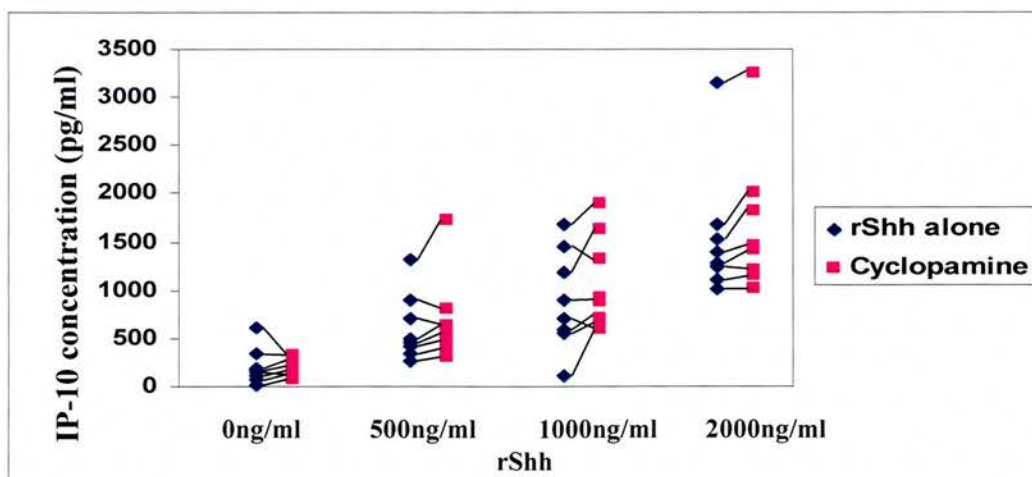


FIGURE 5.11

**Cyclopamine fails to modulate the rShh-mediated upregulation of RANTES, MIG and IP-10 in macrophages cultured in the presence of serum**

Cyclopamine failed to modulate the rShh-mediated upregulation of RANTES (Figure 5.11a), MIG (Figure 5.11b) and IP-10 (Figure 5.11c) by macrophages. Graphs show data from 8 individual experiment. Means and individual data points are shown.

FIGURE 5.12a

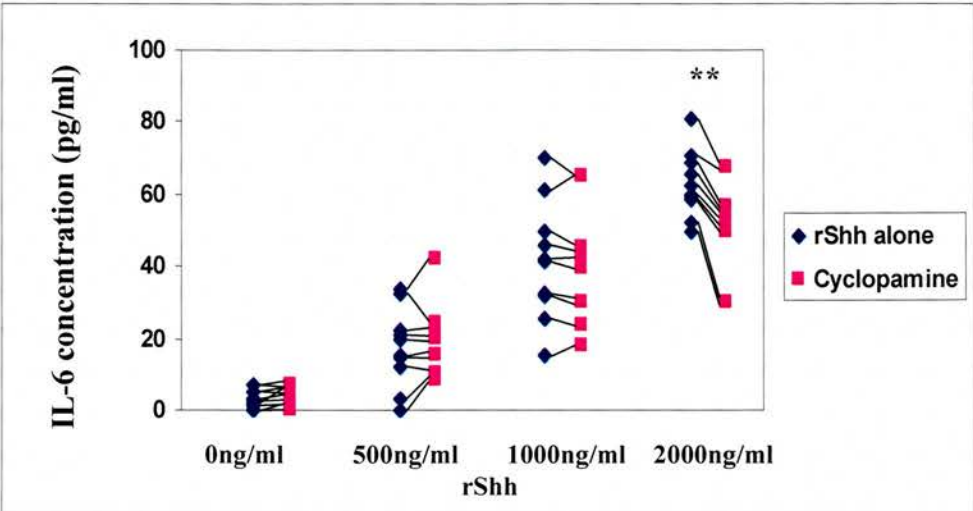


FIGURE 5.12b

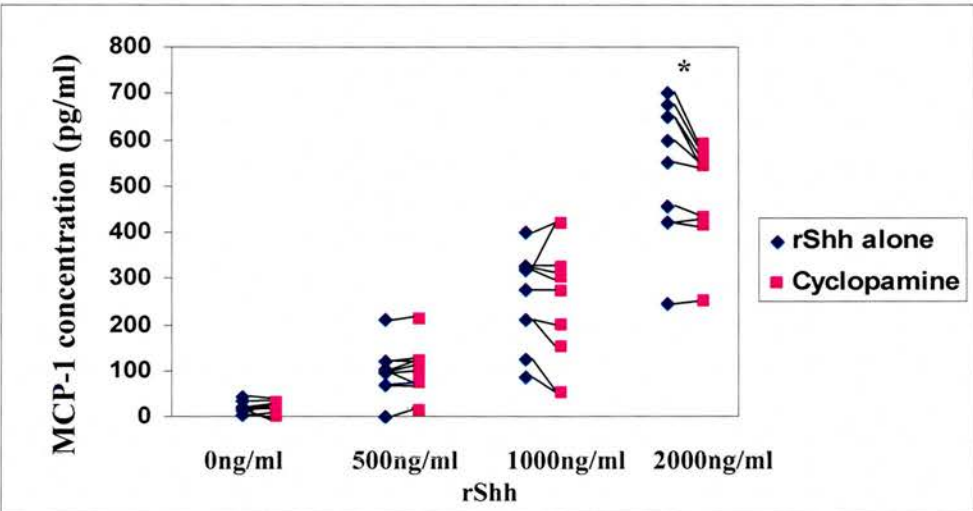


FIGURE 5.12

**Cyclopamine inhibits the rShh-mediated upregulation of IL-6 and MCP-1 in macrophages cultured in the absence of serum**

Macrophages were pre-incubated with 10 $\mu$ M cyclopamine for 45 minutes. rShh was added to the medium to give a final concentration 500-2000ng/ml. As previously, supernatants were harvested for cytokine and chemokine analysis by CBA. Represented are the results from 10 individual donors. The rShh-mediated upregulation of IL-6 (Figure 5.12a) and MCP-1 (Figure 5.12b) was inhibited by cyclopamine at the higher concentrations of rShh used. For the purposes of statistical analysis, cyclopamine treated macrophages were compared to rShh-alone controls using a two-tailed paired t-test. The paired data points are shown. A p value of <0.05 was considered significant (\* p<0.05, \*\* p<0.01, \*\*\* p<0.001).



FIGURE 5.13a

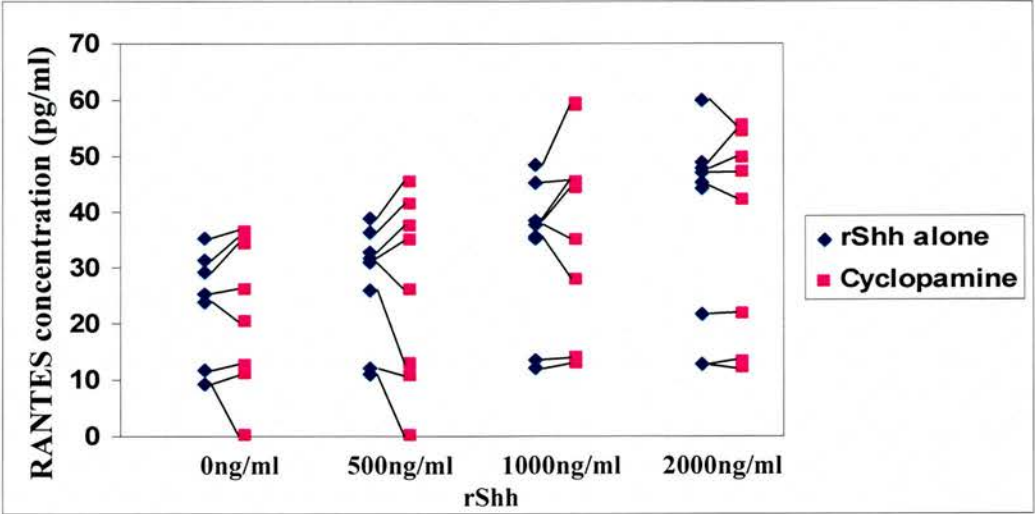


FIGURE 5.13b

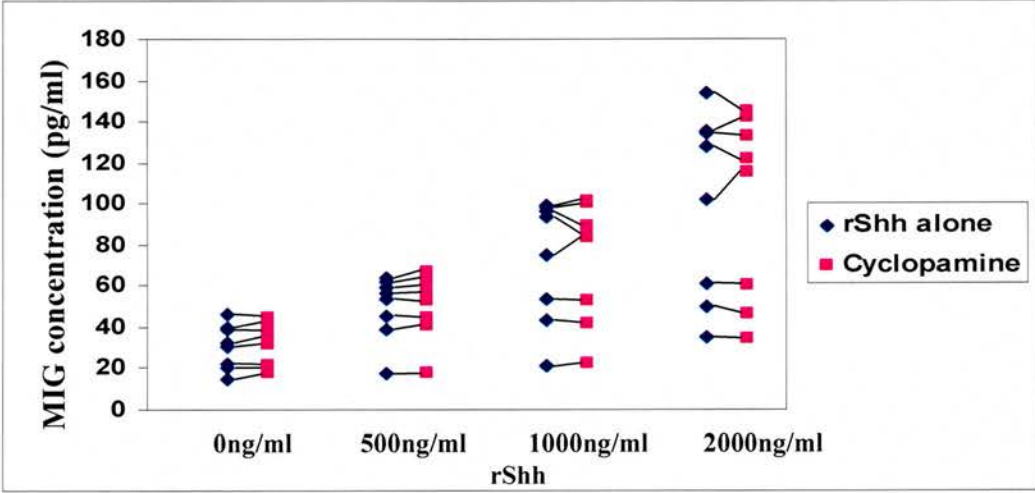


FIGURE 5.13c

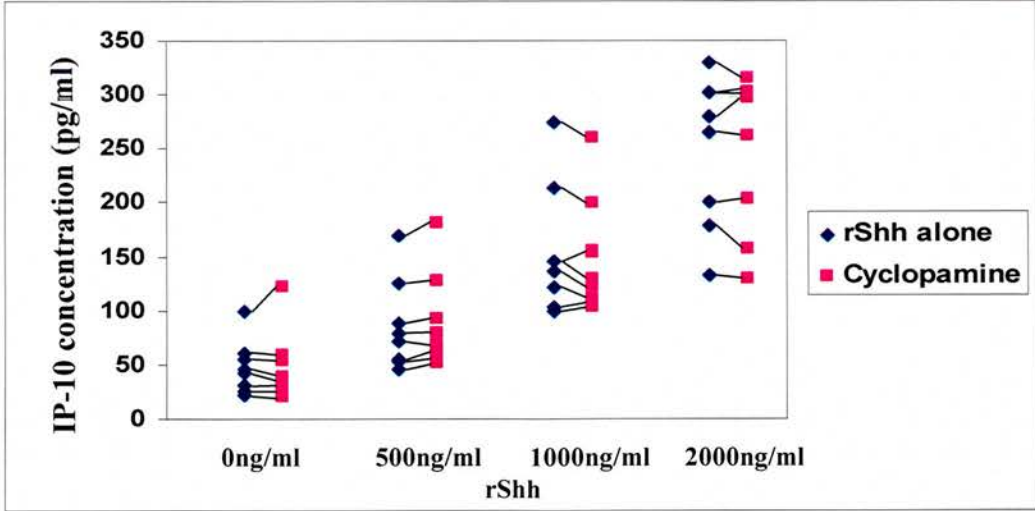


FIGURE 5.13

**Cyclopamine fails to modulate the rShh-mediated upregulation of RANTES, MIG and IP-10 in macrophages cultured in the absence of serum**

Represented are the results from 8 individual donors. Cyclopamine failed to modulate the rShh-mediated upregulation of RANTES (Figure 5.13a), MIG (Figure 5.13b) and IP-10 (Figure 5.13c) by macrophages.

## 5.5 Inhibition of CD14 modulates the macrophage response to rShh

Given recent data suggesting that Shh may be upregulated in epithelium at sites of inflammation<sup>149</sup> and that it may contribute to the development of a proinflammatory cytokine milieu at sites of proliferating T cells<sup>20</sup>, it was tempting to speculate that Shh may be acting as a type of danger signal in keeping with the theory Polly Matzinger put forward in the 1990s<sup>477,564</sup>. This proposed that the immune system responds to endogenous signals originating from stressed or injured cells. Necrotic, but not apoptotic cells, have been shown to activate dendritic cells turning them into powerful APCs<sup>565</sup>. Endogenous signals implicated as possible danger signals have included the heat shock proteins (HSPs)<sup>476,566,567</sup>, including HSP60 and 70, which have been reported to activate macrophages and dendritic cells, enhancing cytokine and chemokine production and upregulating costimulatory molecules<sup>323,568-571</sup>.<sup>291,300,572</sup> That Shh may be upregulated in damaged epithelium and that Shh induces the production of proinflammatory cytokines and chemokines made its possible action as a danger signal an attractive possibility. Recently, a body of evidence has emerged to suggest that the HSPs, HSP 60 and 70 exert their stimulatory effects on macrophages through their action on Toll-like receptor 4 (TLR 4) activation. TLRs are responsible for the recognition of pathogen associated molecular patterns (PAMPs)<sup>300,305,572,573</sup> and, of the 10 TLRs so far described, TLR4 has attracted the most attention. Not only is it the key transducer of the potent bacterial lipopolysaccharide (LPS) signal, but also, it has been proposed to be important in the signal transduction of a wide array of seemingly structurally and functionally unrelated agonists. These include the HSPs 60<sup>319,320,570,574</sup> and 70<sup>322-324,575</sup> noted above, and a wide range of other structurally and functionally unrelated endogenous agonists<sup>576-581</sup>. Signalling through TLR4 induces the activation of the NFkB pathway, the MAP kinases and phosphatidylinositol-3'-kinase (PI-3-K), subsequently resulting in proinflammatory gene transcription and cell-type specific activation phenotypes<sup>291,300</sup>, enhancing antigen presenting activity and providing a key role in linking innate and acquired immune responses<sup>305</sup>.



If indeed Shh is acting as a danger signal, how does it act? In *Drosophila*, there is some evidence that the Toll and Hh signalling pathways may interact. Chen et al<sup>582</sup> demonstrated the expression of a *Drosophila* homologue of CREB-binding protein and posited that it may act as a positive activator of both Toll and Hh pathways. Thus, it is conceivable that the interaction of Shh with Ptc could stimulate similar cellular responses to those seen with Toll pathway activation. A further possibility is that Shh may be mediating its effect on macrophage cytokine and chemokine effector function through TLR4 itself in a similar manner to that proposed for the HSPs. Signalling through TLR4 requires a number of accessory molecules. MD-2 is a small molecule, coexpressed with TLR4 and appears to be essential for signalling<sup>583</sup>. Other important players include LPS-binding protein (LBP), a plasma protein that delivers agonists to the TLR4 signalling complex<sup>584</sup> and CD14, a myeloid marker antigen<sup>585</sup> which can also be found in a soluble form in plasma (sCD14). LBP and CD14 are both components of serum and thus represent candidates which may be responsible for the enhanced macrophage response seen in the presence of serum in the experiments presented in this chapter. CD14 was chosen for initial investigation since reports by other investigators suggested that other proposed endogenous activators of TLR4, HSP60 and 70 can use CD14 to induce the generation of proinflammatory cytokines<sup>323,570,586</sup>. Thus, the aim of these experiments was to determine whether CD14 was important for the Shh-mediated upregulation of macrophage cytokines and chemokines.

Macrophages were pre-incubated for 45 minutes with the purified CD14 functional blocking antibody 61D3 (eBioscience, San Diego, USA) or its isotype control (functional grade purified mouse IgG1 isotype (eBioscience)). The antibody was used in the concentration range 1-100ug/ml as described previously<sup>587-589</sup> and the isotype control was used at 100µg/ml. rShh was subsequently added to the medium to give a final concentration of 1000ng/ml and the supernatants harvested at 24 hours. As previously, the experiments were performed both in the presence and absence of serum.

As shown in Figure 5.14 and 5.15, the antibody successfully and consistently abrogated all cytokine and chemokine responses induced by rShh, both in the presence (Figure 5.14) and absence (Figure 5.15) of serum in all 5 donors studied, suggesting that the effect mediated by Shh appears to be dependent largely on CD14. The effect was maximal at the highest concentration of antibody used. Gross cell morphology was not altered by the antibody and, furthermore, the antibody did not appear to be toxic to the cells as determined using the MTT assay (Figure 5.16).

FIGURE 5.14a

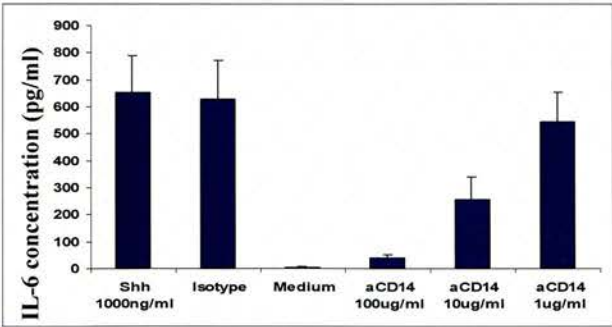


FIGURE 5.14b

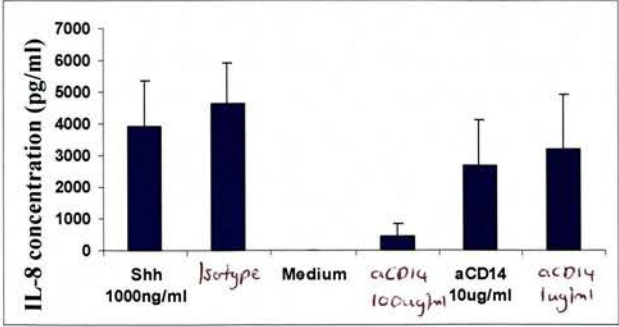


FIGURE 5.14c

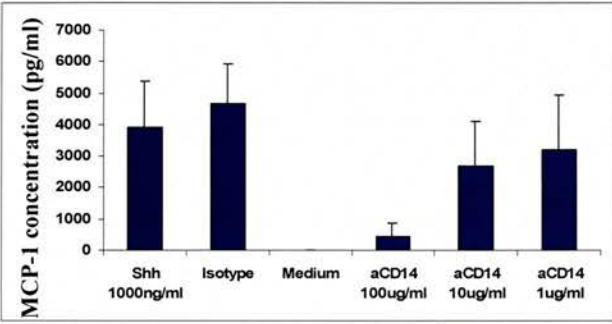


FIGURE 5.14d

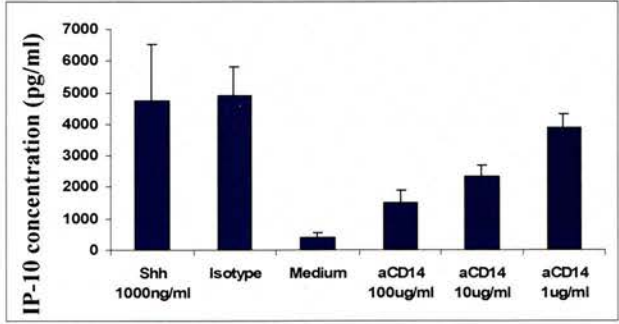


FIGURE 5.14e

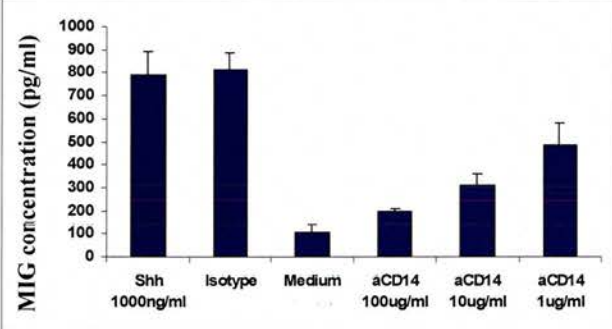


FIGURE 5.14f

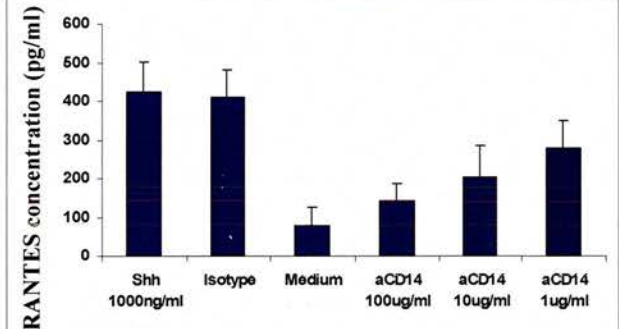


FIGURE 5.14

**CD14 blockade inhibits the rShh-mediated upregulation of IL-6, IL-8, MCP-1, IP-10, MIG and RANTES by macrophages cultured in the presence of serum**

Macrophages were preincubated in the presence of a functional anti-CD14 blocking antibody (aCD14) in the concentration range 1-100µg/ml or an isotype control at 100µg/ml. rShh was subsequently added to the medium to give a final concentration of 1000ng/ml. The left hand column of each graph represents macrophages incubated with 1000ng/ml rShh in the absence of blocking antibody or isotype. Supernatants were harvested at 24 hours and analysed by CBA as previously. CD14 blockade abrogated the production of IL-6 (Figure 5.14a), IL-8 (Figure 5.14b), MCP-1 (Figure 5.14c), IP-10 (Figure 5.14d), MIG (Figure 5.14e) and RANTES (Figure 5.14f) by macrophages in a dose-dependent manner. Represented are pooled results from 5 individual donors. Means and standard deviations are shown.

FIGURE 5.15a

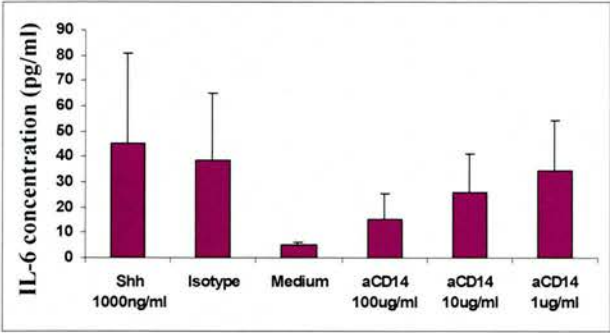


FIGURE 5.15b

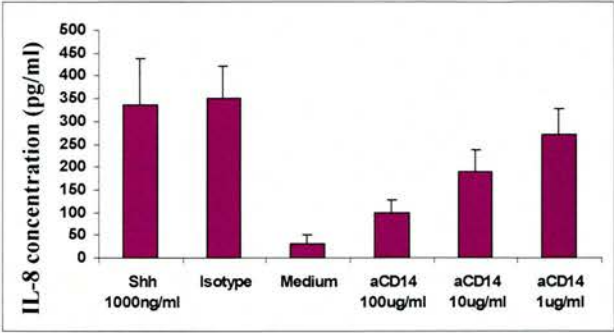


FIGURE 5.15c

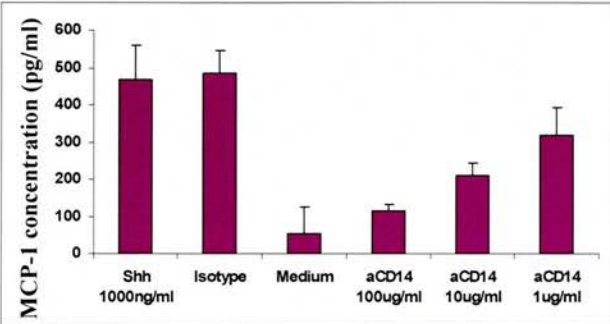


FIGURE 5.15d

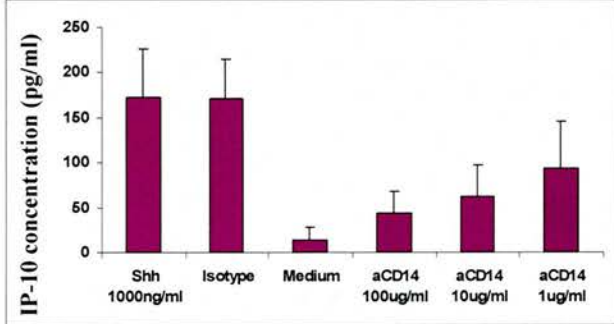


FIGURE 5.15e

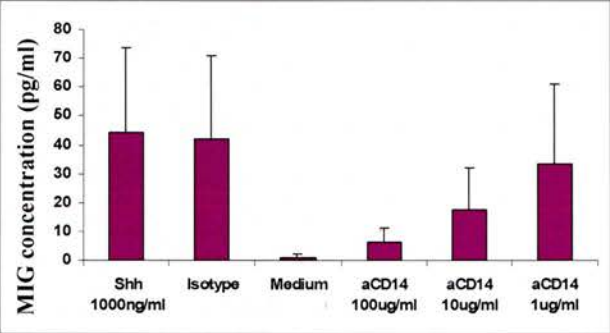


FIGURE 5.15f

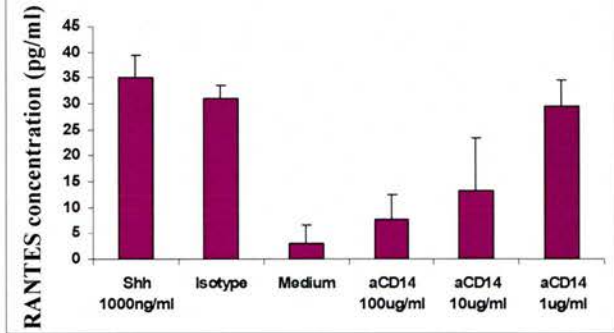
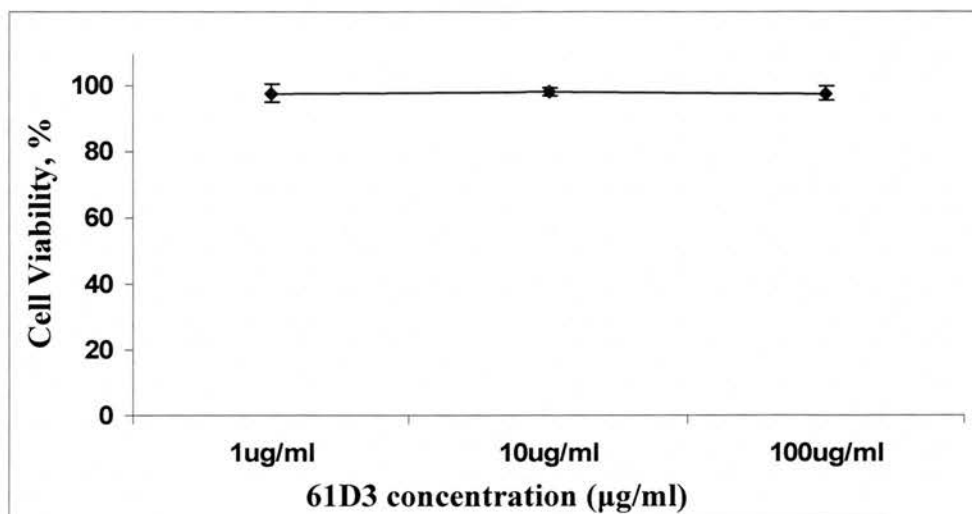


FIGURE 5.15

**CD14 blockade inhibits the rShh-mediated upregulation of IL-6, IL-8, MCP-1, IP-10, MIG and RANTES by macrophages cultured in the absence of serum**

Macrophages were preincubated in the presence of a functional anti-CD14 blocking antibody (aCD14) in the concentration range 1-100µg/ml or an isotype control at 100µg/ml. rShh was subsequently added to the medium to give a final concentration of 1000ng/ml. The left hand column of each graph represents macrophages incubated with 1000ng/ml rShh in the absence of blocking antibody or isotype. Supernatants were harvested at 24 hours and analysed by CBA as previously. CD14 blockade abrogated the production of IL-6 (Figure 5.14a), IL-8 (Figure 5.14b), MCP-1 (Figure 5.14c), IP-10 (Figure 5.14d), MIG (Figure 5.14e) and RANTES (Figure 5.14f) by macrophages in a dose-dependent manner. Represented are pooled results from 5 individual donors. Means and standard deviations are shown.

**FIGURE 5.16**



**FIGURE 5.16**

**Evaluation of the functional CD14 blocking antibody, 61D3, on macrophages adhered on plastic for 6 days.**

Macrophages were pre-incubated with the CD14 functional blocking antibody, 61D3, prior to the addition of rShh (to give a final concentration of rShh of 1000ng/ml). The antibody was used in the concentration range 1-100µg/ml in keeping with previous studies. The cells were incubated for 24 hours in the presence or absence of the 61D3. Viability at the end of 24 hours was evaluated using the MTT assay. Results are presented as the ratio of 61D3-treated to untreated cells (macrophages incubated in 1000ng/ml rShh alone) as a percentage where untreated cells are given a value of 100%. Experiments were performed in triplicate and means and standard deviations are shown.



## 5.6 Endotoxin Considerations

The results from the experiments outlined above suggest that rShh activates macrophages, that this effect is enhanced by serum, differentially modulated by the Shh pathway inhibitor cyclopamine and that CD14 is important in mediating the macrophage effects seen. Given the dependence of TLR signalling on CD14, together these results would implicate the TLRs, either TLR4 and/or TLR2 both of which are dependent to some extent on CD14<sup>307,590-593</sup>, in the mechanism of rShh-induced macrophage activation.

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram negative bacteria, is the most potent TLR4 agonist known and is capable of inducing a wide array of cytokines and chemokines at concentrations as low as 10pg/ml<sup>342,594,595</sup>. Given that the recombinant Shh protein used for these experiments was produced in an *Escherichia coli* recombination system, it was important to detect and quantify any endotoxic contaminants present which could contribute to the activity of the rShh. Inherently, recombinant proteins potentially contain low levels of a number of bacterial products. These can include LPS itself, but also bacterial DNA, and outer wall proteins such as porins and lipid A-associated proteins, fimbrial proteins, protein A and lipoproteins (reviewed by Henderson et al<sup>596</sup>) all of which are capable of inducing cellular activation, via a range of TLRs, including TLR4, TLR2 and TLR9<sup>597-600</sup>. The rShh ligand used during the present series of experiments was reported by the manufacturer to contain no more than 1EU/μg endotoxin.

The aim of the following experiments was to quantify the endotoxin activity present in the rShh used and, further, to determine the significance of any endotoxic activity present in light of the findings to date.



### 5.6.1 Endotoxin quantification

The *Limulus* Amebocyte Lysate (LAL) assay is the gold standard for LPS detection and quantification. The assay uses a property of amebocyte extracts prepared from the horseshoe crab *Limulus polyphemus* which, when in contact with LPS, induce a series of enzymatic reactions, resulting in gelation of the lysate<sup>601</sup>. The early assays relied on the development of a visible clot. Further advances in LAL technology have resulted in the development of systems adapted for instrumental use, including turbidometric and chromogenic methods, which can be either endpoint (fixed incubation time) or kinetic assays (rate of increase of turbidity/colour)<sup>602</sup>.

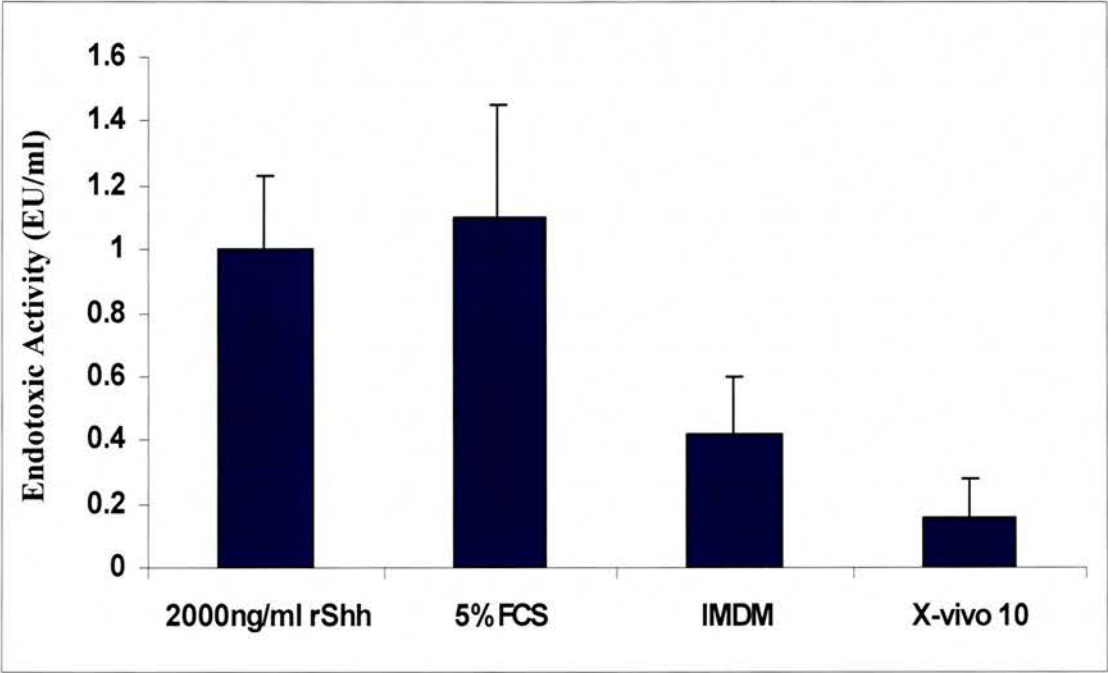
Two approaches were used to determine the endotoxic activity of reagents used in this chapter.

#### 5.6.1.1 LAL chromogenic endpoint assay

To initially evaluate the LPS content of reagents used in these experiments, all reagents were tested using a LAL endpoint chromogenic assay (Associates of Cape Cod, Falmouth, Massachusetts, USA). This chromogenic assay uses a modified LAL reagent (Pyrochrome) incorporating an aqueous extract of the amebocytes of *Limulus polyphemus* and a chromogenic substrate<sup>423</sup>, cleavage of which releases the yellow substance p-nitroaniline which absorbs at 405nm. The amount of p-nitroaniline released is assayed after a fixed incubation time. The results obtained from the samples are plotted against a standard curve consisting of measured optical density plotted against known standard endotoxin concentrations.

The following samples were tested using the assay; 2000ng/ml rShh reconstituted in LAL reagent water (LRW), 5% fetal calf serum reconstituted in LRW, IMDM medium without FCS and X-vivo 10. None of the samples had been stored or frozen and all were prepared using the same stock reagents.

Using this method, 2000ng/ml rShh reconstituted in LAL reagent water was shown to contain approximately 1EU/ml endotoxic activity although, as shown in Figure 5.17, there was marked variation in the values obtained (range 0.75 – 1.50EU/ml). As shown, quantification of the 5% FCS, IMDM and X-vivo 10 also yielded variable results with 1.18EU/ml (range 0.80 – 1.45EU/ml), 0.42EU/ml (range 0.21-0.60EU/ml) and 0.17EU/ml (range 0.05-0.28EU/ml) endotoxic activity respectively. For all test samples, although there was good reproducibility between the triplicates of each sample run on an ELISA plate, significant variability was demonstrated between freshly prepared samples of each and, furthermore, marked inter-plate variability was observed with the same sample run on separate ELISA plates in parallel experiments.



**FIGURE 5.17**

**rShh contains endotoxic activity**

Samples of 2000ng/ml rShh reconstituted in LAL reagent water (LRW), 5% FCS reconstituted in LRW, IMDM and X-vivo 10 were tested for endotoxic activity using a LAL endpoint chromogenic assay and plotted against a standard curve. The results represent pooled data from 10 individual tests. Means and standard deviations are shown.

#### 5.6.1.2 LAL kinetic assay

The LAL assay used in section 5.7.1.1 was a chromogenic endpoint assay. Although easy to use, such assays are reported to lack sensitivity<sup>603-605</sup> and, furthermore, inaccurate results can be achieved when either coloured samples (such as medium containing phenol red), samples containing serum (such as 5% FCS) or those with increased turbidity, are used<sup>604,605</sup>. It is possibly for these reasons that such a wide variation was seen in the results obtained using the endpoint assay.

To partly circumvent the possible pitfalls of using the endpoint assay, samples were also sent to an external endotoxin testing service (Cambrex). The service tests the samples, undiluted and at dilutions of 1:10, 1:100 and 1:100 and uses either of the sensitive chromogenic or turbidometric kinetic assays to quantify the endotoxin contained within the samples.

Using this service, 2000ng/ml rShh reconstituted in LRW was found to contain an average of 1EU/ml endotoxic activity (range 0.9-1.1EU/ml) consistent with the average results from the in-house assay. 5% FCS contained 1.15EU/ml endotoxic activity (range 0.75-1.32EU/ml) and IMDM and X-vivo 10 both contained less than 0.05EU/ml endotoxic activity (range 0.01-0.07EU/ml).

#### **5.6.2 The administration of LPS with equivalent endotoxic activity does not induce the same magnitude of response as rShh**

Given that 2000ng/ml rShh reconstituted in LRW contains 1EU/ml endotoxic activity, the following experiments were set up to determine whether *E coli* LPS of equivalent endotoxic activity induced the same changes in macrophage effector function as those seen in response to rShh.

*E coli* 055:B5 LPS (Sigma) was reconstituted in LAL reagent water to give a standard range of LPS concentrations 15.6-1000pg/ml. Aliquots of these standards were sent to Cambrex for endotoxin testing as previously. Using this LPS

preparation, 125pg/ml LPS in LAL reagent water returned the same level of endotoxic activity (1EU/ml) as 2000ng/ml rShh reconstituted in LAL reagent water. Thus, in parallel experiments, rShh and LPS were reconstituted in either IMDM-FCS or X-Vivo 10 to give equivalent levels of endotoxic activity. Macrophages were then prepared from PBMCs as for all previous experiments. On day 6, the macrophages were stimulated with either 2000ng/ml rShh or 125pg/ml LPS. The experiments were performed in 5 donors using the same stock titrations of LPS and the same stock preparation of rShh. The results of these experiments are illustrated in Figure 5.18 and Figure 5.19 and, together, suggested that rShh induced an upregulation in cytokines which exceeded that induced by LPS of equivalent endotoxic activity.

FIGURE 5.18a

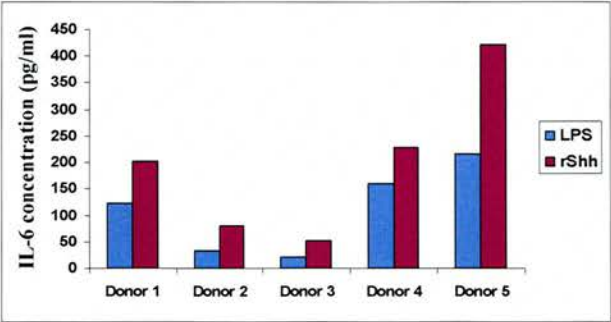


FIGURE 5.18b

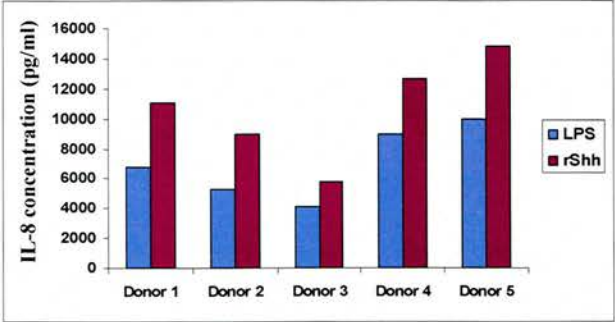


FIGURE 5.18c

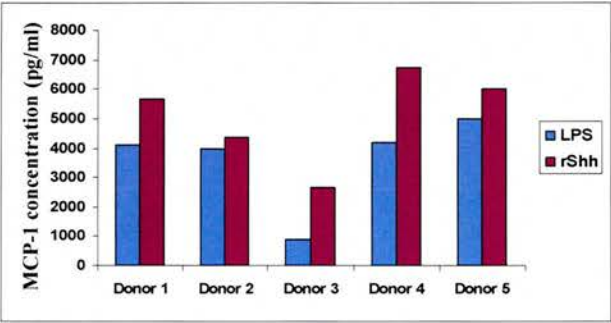


FIGURE 5.18d

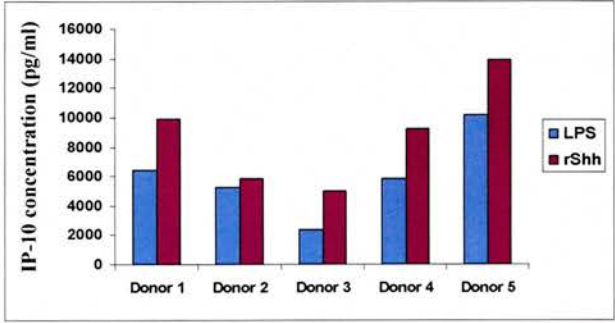


FIGURE 5.18e

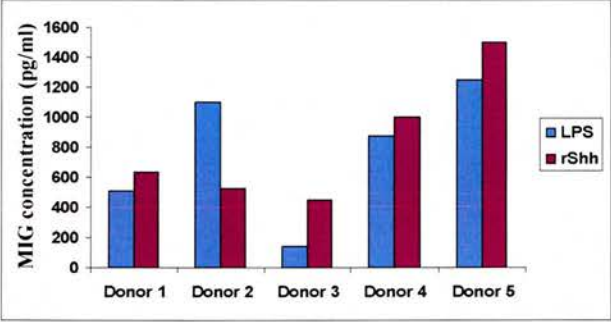


FIGURE 5.18f

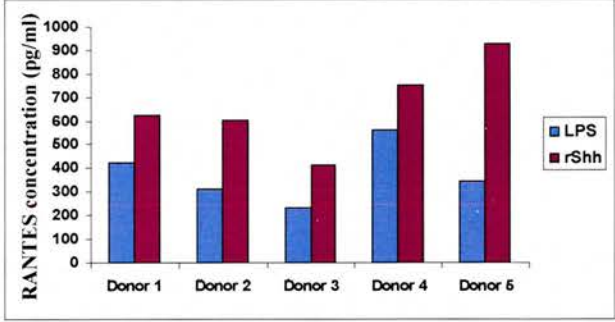


FIGURE 5.18

**rShh induces a greater upregulation in cytokines and chemokines compared to LPS of equivalent endotoxic activity in the presence of serum**

Macrophages were cultured either in 2000ng/ml rShh or *E coli* LPS of equivalent endotoxic activity. Pooled results from 5 donors for each cytokine or chemokine are shown. rShh induced an upregulation in IL-6 (Figure 5.18a), IL-8 (Figure 5.18b), MCP-1 (Figure 5.18c), IP-10 (Figure 5.18d), MIG (Figure 5.18e) and RANTES (Figure 5.18f) exceeding that induced by LPS of equivalent activity.



FIGURE 5.19a

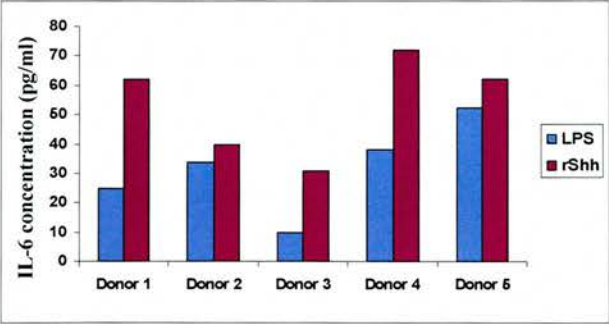


FIGURE 5.19b

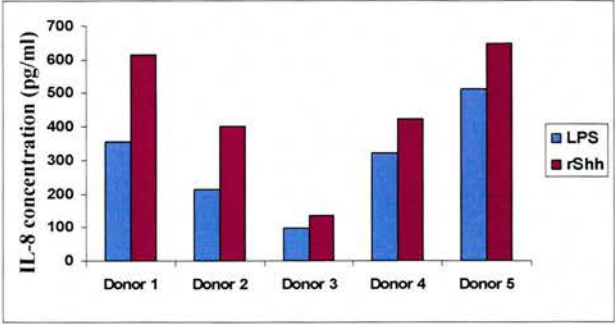


FIGURE 5.19c

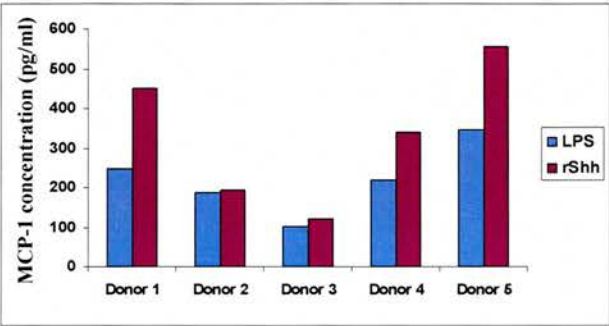


FIGURE 5.19d

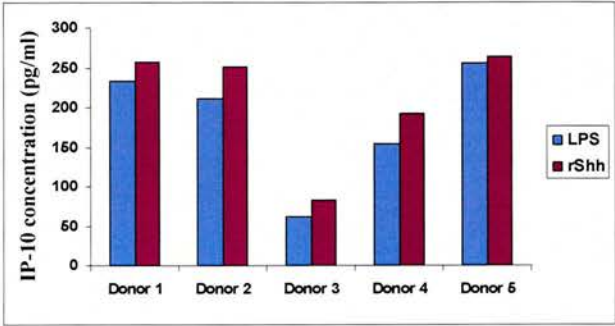


FIGURE 5.19e

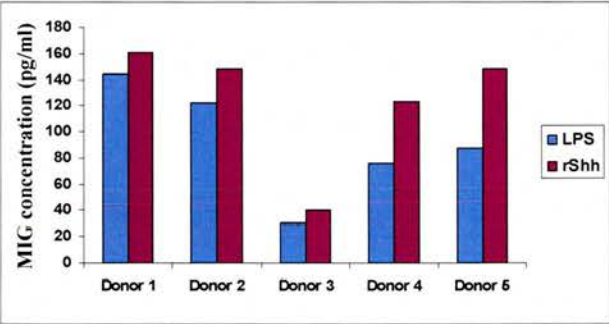


FIGURE 5.19f

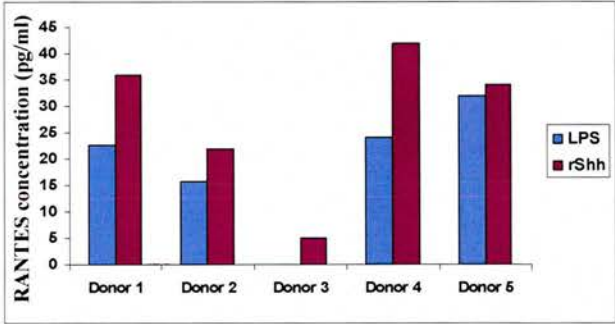


FIGURE 5.19

**rShh induces a greater upregulation in cytokines and chemokines compared to LPS of equivalent endotoxic activity in the absence of serum**

Macrophages were cultured either in 2000ng/ml rShh or *E coli* LPS of equivalent endotoxic activity. Pooled results from 5 donors for each cytokine or chemokine are shown. rShh induced an upregulation in IL-6 (Figure 5.19a), IL-8 (Figure 5.19b), MCP-1 (Figure 5.19c), IP-10 (Figure 5.19d), MIG (Figure 5.19e) and RANTES (Figure 5.19f) exceeding that induced by LPS of equivalent activity.

## 5.7 Polymyxin B abrogates the macrophage response to rShh

Although rShh induced greater changes in cytokine production than those induced in response to LPS in all donors, these changes were observed to fall within the margins of error incorporated within the endpoint LAL assay (50-200%). Thus to further exclude the possibility that endotoxin may be contributing to the effects on cytokine and chemokine production seen, it was necessary to adopt a different approach to differentiating the effects of Shh from any endotoxin contamination present within the protein preparation.

The following experiments were based on the use of an LPS neutralising agent, polymyxin B (PMB). PMB is a polycationic peptide antibiotic that binds to the anionic lipid A portion of LPS and neutralises its endotoxic activity<sup>606-608</sup>. PMB has been used widely in the literature<sup>315,323,421,566,609-612</sup> to differentiate between effects due to the protein of interest from those of endotoxin contamination and, although not active against all species of LPS<sup>613</sup>, is known to be effective against *E coli* LPS.

Two experimental approaches were used to attempt endotoxin neutralisation. The first utilised immobilised PMB in the form of PMB-agarose beads (Sigma) and the second used PMB as a culture additive.

### 5.7.1 The use of PMB-agarose beads to remove contaminating LPS

Immobilised PMB adsorption has been used previously in the literature to remove contaminating endotoxin from a wide variety of solutions<sup>611,612,614-616</sup>. In the present set of experiments, the following samples were incubated with PMB agarose beads for a period of 30 minutes at 37 degrees: 2000ng/ml rShh reconstituted in LRW, 10µg/ml and 100pg/ml *E coli* LPS. Samples of each were set aside for later LAL testing prior to incubation with PMB-coated beads and, furthermore, a sample of 2000ng/ml rShh was set aside for protein assay. Following incubation with the PMB beads, the mix was centrifuged at 300g to precipitate out the beads. The supernatants

were then removed and assayed for endotoxic activity and, in the case of the rShh, for protein content. Whilst PMB beads successfully removed most of endotoxic activity from 10µg/ml LPS, it failed to remove that from either the 100pg/ml LPS or indeed the rShh preparation. Furthermore, significant protein losses in the order of 50% were observed when samples were analysed in a Bradford protein assay (data not shown), both findings in keeping with other reports in the literature<sup>609,610,615,617-620</sup>. In view of these results, other methods had to be employed to attempt endotoxin removal.

### 5.7.2 The use of Polymixin B as a culture additive

The problem of protein adsorption has been circumvented in previous studies by using PMB as a culture additive<sup>323,421,569,581,609,610,612,621-625</sup>. Although the recombinant protein is still tightly adherent to LPS, the premise is that, whilst PMB neutralises the lipid A component of the LPS it still allows the protein to exert its effect in culture. Therefore, the following experiments employed PMB as a culture additive to see whether it modulated the effect of rShh on macrophage effector response. PMB was used at a concentration of 10µg/ml as previously described in a large number of *in vitro* studies involving monocytes, macrophages and dendritic cells<sup>569,581,609,610,625</sup>.

PMB (10µg/ml) was added to all macrophage cultures 45 minutes prior to the addition of the stimulating agent (rShh or LPS 100ng/ml) and the cells were incubated under these conditions for 24 hours. PMB was also added to macrophage cultures following the addition of cyclopamine and prior to stimulation with rShh to see whether PMB could modulate the cyclopamine-mediated inhibition of the rShh-induced macrophage activation. Supernatants were harvested and analysed using the cytokine and chemokine CBAs as above.

As demonstrated in Figures 5.20 and 5.21, PMB profoundly abrogated the response of macrophages to rShh at all concentrations used and in all donors tested (n=8).

These results suggest that the dominant effect of rShh on macrophages is induced by the LPS contained within the recombinant protein preparation.

FIGURE 5.20a

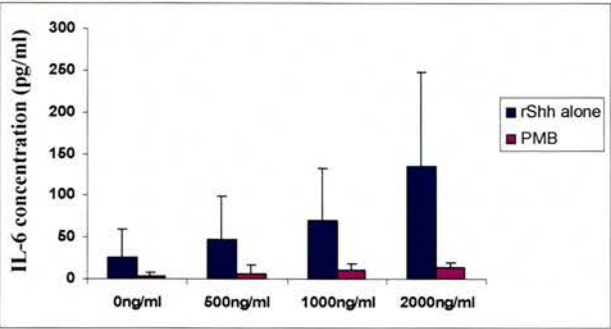


FIGURE 5.20b

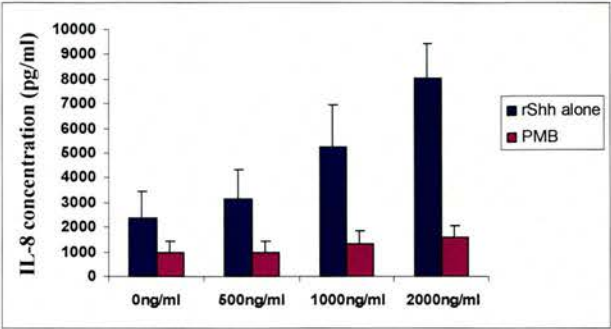


FIGURE 5.20c

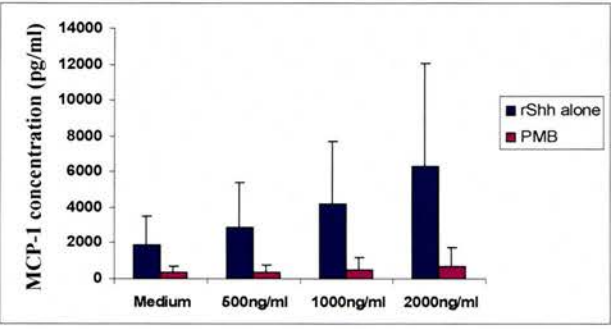


FIGURE 5.20d

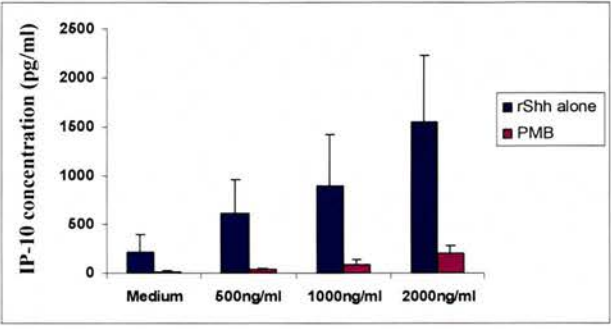


FIGURE 5.20e

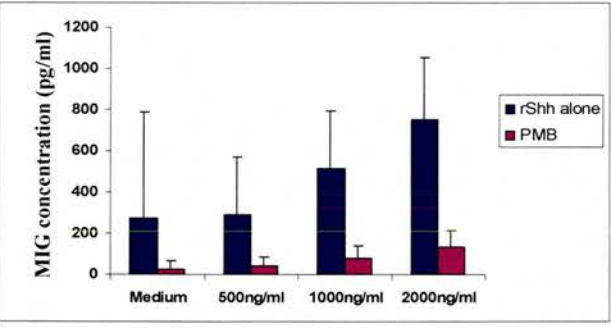


FIGURE 5.20f

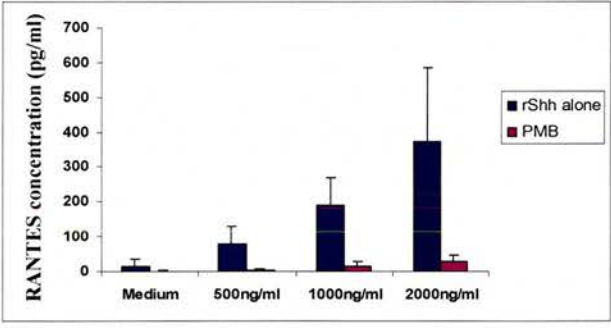


FIGURE 5.20

**Polymixin B abrogates the rShh-mediated upregulation of cytokines and chemokines in the presence of serum**

Macrophages were preincubated in the presence of 10μg/ml PMB prior to the addition of rShh in the concentration range 500-2000ng/ml. Supernatants were harvested at 24 hours and analysed by CBA as previously. PMB markedly inhibited the production of IL-6 (Figure 5.2-a), IL-8 (Figure 5.20b), MCP-1 (Figure 5.20c), IP-10 (Figure 5.20d), MIG (Figure 5.20e) and RANTES (Figure 5.20f) by macrophages in a dose-dependent manner. Represented are pooled results from 8 donors. Means and standard deviations are shown.



FIGURE 5.21a

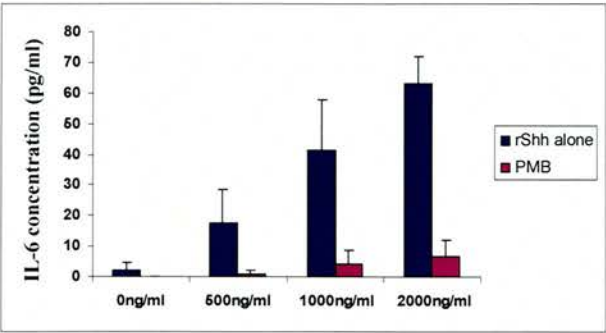


FIGURE 5.21b

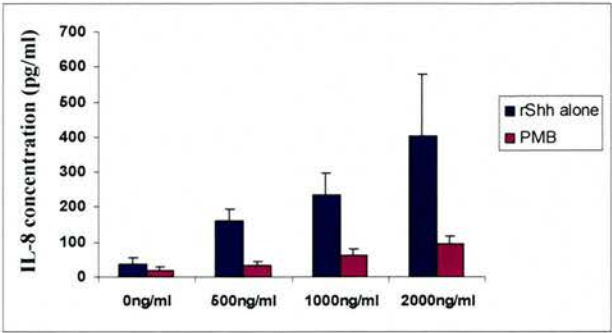


FIGURE 5.21c

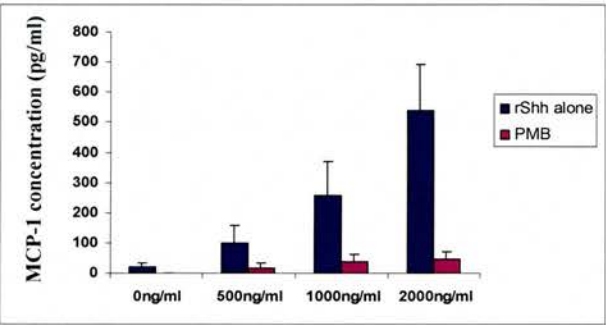


FIGURE 5.21d

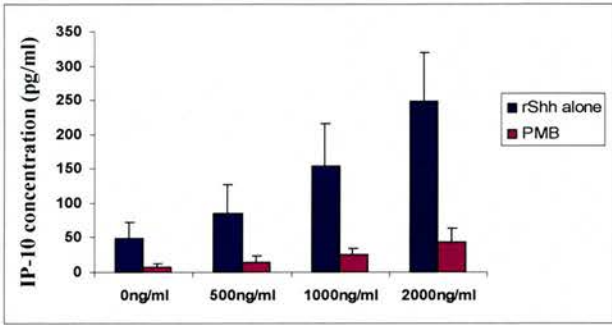


FIGURE 5.21e

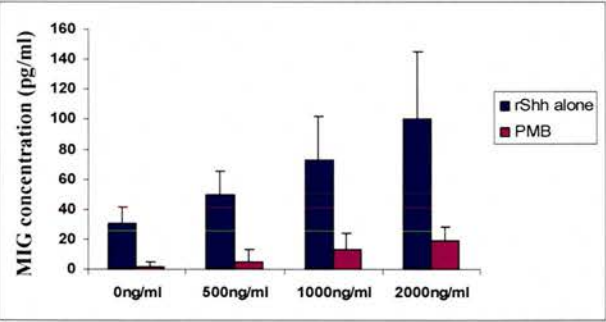


FIGURE 5.21f

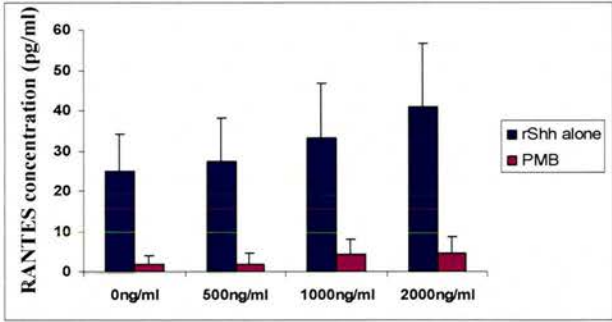


FIGURE 5.21

**LPS neutralisation using Polymixin B abrogates the rShh-mediated upregulation of cytokines and chemokines in the absence of serum**

Macrophages were preincubated in the presence of 10µg/ml PMB prior to the addition of rShh in the concentration range 500-2000ng/ml. Supernatants were harvested at 24 hours and analysed by CBA as previously. PMB markedly inhibited the production of IL-6 (Figure 5.21a), IL-8 (Figure 5.21b), MCP-1 (Figure 5.21c), IP-10 (Figure 5.21d), MIG (Figure 5.21e) and RANTES (Figure 5.21f) by macrophages in a dose-dependent manner. Represented are the pooled results from 8 donors. Means and standard deviations are shown.

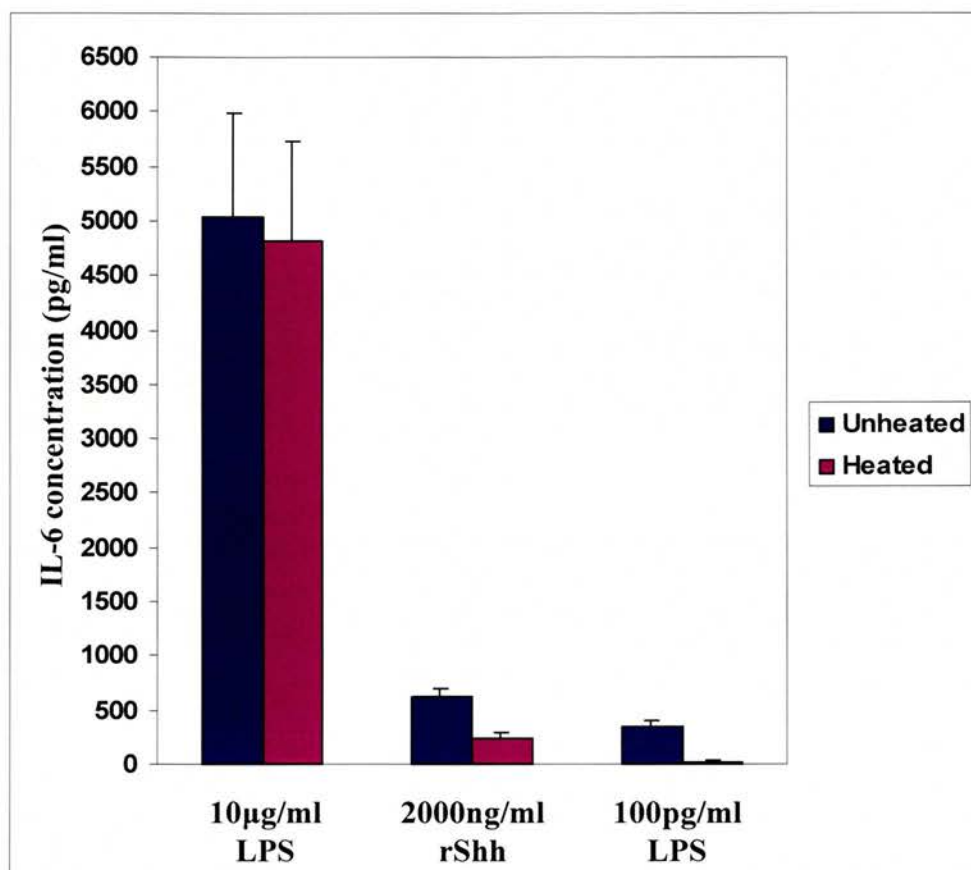


## 5.8 rShh and low concentrations of LPS are sensitive to heat

Classically, LPS has been considered to be heat resistant<sup>322-324,575,626</sup> and this property has been used extensively in the literature to differentiate the effects of a protein of interest from the effects of any LPS contamination contained within the protein<sup>320-324,566,570,575,576,581</sup>. More recently, the use of this property to distinguish LPS effects from those due to a protein of interest has been called into question following recent reports suggesting that low concentrations of LPS may be very heat sensitive<sup>609,610</sup>.

Thus, the following experiments were performed to determine whether heating rShh would modulate the macrophage cytokine and chemokine response. For controls, 10µg/ml and 100pg/ml *E coli* LPS were used in parallel experiments. Samples of each were heated for 1 hour at 100 degrees in a heating block as previously described<sup>609,610</sup>. Macrophages were then cultured in the presence of both untreated and heat-treated 2000ng/ml rShh, 10µg/ml LPS and 100pg/ml LPS.

Figure 5.22 shows the effect of these treatments on IL-6 production as an example. As shown, the macrophage response to 10µg/ml LPS was preserved following heating, a finding consistent with previous reports. In contrast, heat-treatment abrogated both the response of macrophages to 2000ng/ml rShh and 100pg/ml LPS. Similar results were seen irrespective of the cytokine or chemokine examined. These results are in keeping with recent data suggesting that small quantities of *E coli* LPS are heat sensitive<sup>609,610</sup>.



**FIGURE 5.22**

**rShh and low concentrations of LPS are heat-sensitive**

*E coli* LPS (10µg/ml and 100pg/ml) and rShh (2000ng/ml) were heated at 100°C for 60 minutes prior to macrophage culture for 24 hours. Supernatants were harvested for cytokine and chemokine analysis as previously. Graph represents IL-6 production from macrophages from 3 donors cultured in medium containing serum. Means and standard deviations are shown.

## 5.9 Discussion

Although Shh signalling components have been previously identified in murine and human macrophages<sup>19,149</sup>, the possible role for Shh in modulating macrophage effector function has not been explored to date. In this chapter, macrophages have been prepared from human Buffy coat-derived PBMCs and used to explore the expression of Shh signalling pathway components and the effect of Shh signalling in these cells.

The findings reported in the chapter indicate that macrophages express components of the Shh signalling pathway and that they may thus be able to respond to a Shh signal. Studies investigating a possible role for Shh in the modulation of cytokine and chemokine expression by these cells have been significantly hampered by the presence of contaminating endotoxin contained within the *E coli*-derived recombinant protein utilised to investigate the role of Shh *in vitro*. Indeed, taken together, the results would suggest that endotoxin contamination rather than Shh *per se* is responsible for most, if not all, of the changes in cytokine and chemokine effector function seen. What follows is a discussion of the results and issues that have arisen from this work.

### 5.9.1 Shh signalling pathway expression in macrophages

The results from the early part of this chapter confirm the findings of previous studies<sup>19,149</sup> suggesting that Shh signalling pathway components are expressed in human macrophages. Stewart et al<sup>149</sup> observed Ptc expression in alveolar macrophages, a finding in keeping with the earlier work of Bhardwaj et al<sup>19</sup>. Bhardwaj et al<sup>19</sup> employed RT-PCR to demonstrate the expression of *Ptc*, *Smo*, *Gli1*, *Gli2* and *Gli3* in primitive haematopoietic progenitor cells (CD34+CD38-Lin-) and the expression of *Ptc* and *Smo* in committed CD33+ myeloid cells isolated from cord blood. The findings of the present work broadly agree with these earlier findings in that Ptc was found to be expressed in mature human macrophages both at the mRNA and protein level. Furthermore, *Smo* expression was observed at the mRNA level

consistent with the work of Bhardwaj et al<sup>19</sup>. However, in contrast to this group's findings, *Gli 1* and *2* mRNA were expressed in the PBMC-derived macrophages and, furthermore, although Bhardwaj et al<sup>19</sup> identified *Shh* expression in both the primitive precursor and committed CD33+ populations, neither *Shh* mRNA nor Shh protein were detected in the PBMC-derived macrophages in the present work. The reason for these differences is not clear. Lineage and maturation-dependent differential gene expression in haematopoietic cells have been described previously for a number of genes including *flt3*<sup>627</sup>, the homeobox genes<sup>628</sup> and the downstream targets of Shh activation, the bone morphogenetic protein(BMP) family<sup>511</sup> and it remains to be seen whether such differences may account for the conflicting results seen.

It is interesting that, although macrophages expressed *Ptc*, *Smo*, *Gli 1* and *Gli 2* at the mRNA level, only *Ptc* was convincingly demonstrable at the protein level. Indeed there are few reports in the literature evaluating the protein expression of Shh pathway components in the peripheral immune system. Stewart et al<sup>149</sup> used the same goat polyclonal antibody as used in this thesis to demonstrate *Ptc* expression on alveolar macrophages and the findings of the present study are in keeping with this report. However, further studies investigating the expression of other components of the pathway have not been performed. Although, on initial evaluation, *Smo*, *Gli1* and Shh appeared to be expressed by macrophages, this staining could not be blocked using the appropriate specific blocking peptides, thus suggesting that the staining was non-specific in nature.

Following the discovery that macrophages expressed the receptor for Shh, *Ptc*, the next line of investigation was to determine whether active Shh signalling could occur in these cells. Indeed, that PBMC-derived macrophages appear to be able to respond to but not produce a Shh signal would be in keeping with studies in the chick embryo where Shh and *Ptc* are often only co-expressed transiently during development<sup>99</sup> later either producing Shh or being able to respond to a Shh signal. In keeping with earlier reports in the literature<sup>8,16,20,32,44,102</sup>, *Ptc* mRNA upregulation was used as a readout of Shh signalling pathway activation. Using real-time PCR, a 2-fold increase was

accepted as representing significant gene upregulation consistent with previous reports<sup>20,507,508</sup> and, in the present studies, an average 2.8-fold increase in *Ptc* expression was observed in response to stimulation with 2000ng/ml rShh. It may be that a lower magnitude of mRNA upregulation is biologically relevant in macrophages as suggested by others<sup>629</sup>. That *Ptc* is upregulated to this extent at 24 hours is in agreement with previous studies in T cells<sup>20</sup> and myofibroblasts<sup>102</sup> and would suggest that active signalling through the Shh signalling pathway occurs. However the significance of this magnitude of upregulation in macrophages is not clear. Furthermore, it is not known whether this upregulation in mRNA translates into protein expression. Such changes are difficult to quantify using immunohistochemistry and further investigation of protein expression are difficult for the reasons noted previously.

Further investigation at the protein level may prove helpful to help delineate whether the Shh signalling does indeed play an active role in macrophages. To date, it has been generally considered that *Ptc* mRNA upregulation specifically reflects active Shh pathway signalling. However, this may not be the case. Preliminary experiments performed recently suggested that LPS too may also regulate *Ptc* expression. Indeed, in experiments in 2 donors, 1-100ng/ml *E coli* 055:B5 LPS (Sigma) induced *Ptc* expression in macrophages after 18 hours in culture (See Appendix 3). This supports earlier observations suggesting that, in murine peritoneal and bone marrow derived macrophages, LPS upregulates *Ptc* (Armenika Etemi, unpublished observations). Taken together, these results would suggest that either *Ptc* upregulation is not specific to the Shh pathway or that LPS signalling and the Shh pathway converge. Interestingly, PI-3-K, a downstream target of LPS signalling<sup>630-632</sup> has been shown recently to be upregulated in response to rShh in endothelial cells<sup>65</sup>. Furthermore, that LPS may regulate components of the Hh signalling pathway has been demonstrated in other recent studies<sup>633</sup>.

### 5.9.2 rShh and macrophage effector function

The early experiments investigating the effect of rShh on macrophages revealed that stimulation of these cells with rShh resulted in the upregulation of a large number of inflammatory mediators including VEGF, IL-6, IL-8, MCP-1, RANTES, IP-10 and MIG, factors also recognised to be upregulated by LPS. Conversely, other factors classically upregulated by LPS, such as IL-1 $\beta$ , IL-12p70, TNF $\alpha$  and IL-10 were not consistently upregulated by rShh. Given that the later results of the chapter support an argument for LPS mediating the dominant macrophage effect, it is not clear why LPS, but not Shh, can induce IL-1 $\beta$ , IL-12p70, TNF $\alpha$  and IL-10. One possible reason for this disparity may reflect the possible heterogeneity of the 2 stimuli being compared. As noted previously, like other recombinant proteins produced in bacterial recombination systems, rShh is likely to contain not just LPS but also a wide range of other bacterial components including lipoproteins and bacterial DNA which are capable of activating several TLRs including TLRs 2, 4 and 9<sup>596-598</sup>. Interestingly, LPS itself rarely exists in a pure form, and is usually found in close association with LPS-associated lipoproteins, which are difficult to eliminate from LPS preparations<sup>596,598</sup>. The importance of such lipoproteins should not be underestimated. Indeed, in the early studies of LPS signalling, it was initially thought that TLR2, and not TLR4, was the TLR responsible for *E coli* LPS signal transduction. It was subsequently demonstrated that activation of TLR2 by commercial LPS disappeared when the LPS was repurified under stringent conditions highlighting the importance of these lipoproteins as misleading contaminants<sup>634-637</sup>. The LPS used for the present studies was of the *E coli* serotype 055:B5 and the protein content of this preparation has been reported to be less than 3%. Thus, there exists the possibility that a small amount of lipoprotein (albeit less than 3%) has the potential to activate the macrophages through TLR2. Furthermore, very little is known regarding the relative contribution of the possible TLR agonists present in the rShh peptide. Given that PMB abrogated, but failed to completely eliminate the rShh-mediated response, it is possible that other TLR agonists present within the recombinant protein, which may include Shh itself, are responsible for the



residual cytokine and chemokine production seen. Thus, it can also be seen that contributions from several TLRs have the potential to significantly modify the macrophage effector response.

Furthermore, the complexities of the TLR signalling pathways are only just beginning to be understood. It was originally considered that all TLRs converged on the same pathways and that there was little specificity between activation events. More recently, however, a body of evidence has evolved to suggest that the pathways downstream of different TLRs are differentially activated by the TLR agonists<sup>346,630,631,638-640</sup>. Complicating matters further, there are reports of cross-talk between different TLRs<sup>641</sup> and, furthermore, there is evidence that TLR agonists may act synergistically to induce inflammatory cytokine production. This has been described for TLR2 and TLR4 agonists<sup>637,638,642-645</sup> and bacterial DNA may also synergise with LPS to induce inflammatory cytokines<sup>639,646,647</sup>. In another interesting report, synergy between the TLRs 2, 5, 7 and 9 and adenosine receptors has been shown to modulate cytokine production resulting in the upregulation of VEGF and downregulation of TNF $\alpha$  production<sup>648</sup>. These findings would concur with the results of the current work. From Pinhal-Enfeld et al's work, it is suggested that this upregulation of VEGF together with the down-regulation of TNF $\alpha$  may act as an angiogenic switch, shifting macrophages from an inflammatory to an angiogenic phenotype. Given the previous reports linking Shh signalling and VEGF signalling<sup>102,163</sup> and, thus, a possible role for Shh in postnatal angiogenesis, it would be very interesting to see whether a pure preparation of Shh was capable of inducing similar changes in macrophages to those seen in this study in response to rShh.

A further possibility has yet to be considered: that synergy may occur between Shh and endotoxic contaminants present within the recombinant protein. Synergy has been shown to exist between endotoxins and TNF $\alpha$ <sup>649</sup>. Given that one possible role for Shh might be to act as a danger signal when epithelium is damaged, it is tempting to speculate that Shh released from such cells might synergise with low levels of LPS present to initiate an immune response to that stress signal. Preliminary experiments were performed to investigate this possibility. Macrophages were stimulated either

with rShh, LPS or a mixture of the two. The results from these preliminary experiments were inconclusive with evidence of both summative effects and reduced cytokine production when both LPS and rShh were present. Interestingly, in no instance in the 3 donors examined was there any evidence of synergy. Furthermore, if cyclopamine and PMB were together added to a macrophage culture prior to the addition of Shh, the inhibitory effects of cyclopamine and PMB were not seen to be additive (Appendix 4). Together these results would suggest that synergy between LPS and Shh is not occurring, although the purity issues outlined above make interpretation of such experiments difficult.

### **5.9.3 The effect of cyclopamine**

Following the discovery that rShh upregulated a number of cytokines and chemokines, the specific inhibitor of the Shh pathway, cyclopamine, was used to determine whether inhibition of signalling modulated the production of these factors. In these studies, cyclopamine had a differential effect on the upregulation of cytokines and chemokines with only the rShh-mediated upregulation of IL-6 and MCP-1 being abrogated significantly in the first 10 donors studied. IL-8 upregulation was inhibited in only some donors. Although these factors were modulated by cyclopamine, most factors that were upregulated by rShh, namely RANTES, IP-10, MIG and VEGF were not affected by the addition of cyclopamine.

The differential effect of cyclopamine in the present work proved an interesting finding. If the effects originally attributed to Shh are in fact due to residual endotoxin contained within the recombinant protein, why does cyclopamine have any effect at all? Differential inhibition of the Shh signalling pathway has been previously reported in which inhibition of Shh signalling inhibited IL-2 and IFN- $\gamma$  production but not IL-10 production from activated T cells<sup>20</sup>. Furthermore, mutations in Smo have been described<sup>23,24</sup> and therefore there is the possibility that these may account for the differential response to cyclopamine. However, a more fundamental issue remains. Although cyclopamine has been reported to act by inhibiting activated Smo, the mechanism by which it does this has not been fully delineated<sup>60,650,651</sup>. Most of

the original work performed using cyclopamine was performed in developmental studies<sup>64,652</sup> and there are fewer reports of its use *in vitro*<sup>65,653</sup>. Thus, although it did not appear to have a toxic effect on the macrophages, as determined using trypan blue exclusion and the MTT assay, that it may have had an effect elsewhere on other macrophage signalling pathways cannot be excluded. To circumvent this possibility, it would be ideal to use another inhibitor of the Shh pathway. In this regard, the monoclonal antibody 5E1, which has been used previously in *in vitro* studies<sup>20,21,147</sup> would appear ideal. Steric interference mapping analysis studies suggested that 5E1 binds to Shh at a site distinct from, but possibly overlapping, the Ptc binding site<sup>654</sup>. However, aliquots of this antibody extracted from the myeloma cell line supernatant grown in-house were found to contain significant amounts of endotoxic activity (in the range 1-5ng/ml) and, thus, were not suitable for use in culture with LPS responsive cells. Another possible reason for the differential inhibition by cyclopamine may lie in the preparation of cyclopamine used. Cyclopamine, provided as a lyophilised pellet was reconstituted in DMSO according to the manufacturer's instructions. This stock concentration was then further diluted in medium to provide further stocks to avoid thawing-refreezing cycles and possible loss of activity of the compound. Of particular interest was the finding that aliquots of the secondary stocks when diluted in LRW were found to contain varying amounts of endotoxic activity (0.25 – 0.50 EU/ml). Given that the LRW was free of endotoxic activity, the origin of this must either be the cyclopamine itself, the DMSO or the tiny quantity of serum that was present in the medium used to prepare the secondary stocks of cyclopamine. Interestingly, when a new batch of cyclopamine was reconstituted in new DMSO and prepared into secondary stocks using LRW, the endotoxic activity of these aliquots was <0.05EU/ml. Recent preliminary experiments using these new stocks in 3 donors have since showed failure of the cyclopamine to inhibit the rShh-mediated IL-6 and MCP-1 production previously observed with the older stocks.

One possible reason for the initial observations then might be that pre-treatment of the macrophages for 45 minutes with cyclopamine containing low concentrations of endotoxin may have desensitised the macrophage to a subsequent stimulus, provided in the form of the rShh applied. Previous reports have demonstrated a state of LPS

hyporesponsiveness induced by pre-treatment with low concentrations of LPS<sup>655,656</sup>. This effect is referred to as tolerance or desensitisation and is characterised by the decreased production of cytokines. The mechanisms responsible for such desensitisation have not been fully delineated. Previous reports in the literature have postulated on the induction of inhibitory mediators<sup>657</sup>, modulation of membrane bound receptors such as CD14<sup>658,659</sup> and changes in the intracellular signal transduction pathway<sup>655</sup> following pre-incubation with low doses of LPS.

The differential temporal regulation of cytokines in response to LPS has been described and upregulation of cytokine mRNA expression occurs early in response to LPS<sup>629,660</sup> for several cytokines. However, whether a 45 minute incubation at such a low concentration of endotoxic activity is sufficient for such endotoxin tolerance or desensitisation to occur is not known. Interestingly, in a recent study, a 90 minute incubation with low dose LPS was sufficient to induce suppressed MAP kinase activity and tolerance to a second LPS stimulus<sup>661</sup>.

If indeed desensitisation plays a role in the effects seen, it is interesting that only some factors (IL-6 and MCP-1) were affected. Recent data supporting such a differential desensitisation has shown that pre-treatment of monocytes with 10-1000pg/ml LPS was sufficient to induce a markedly reduced response in a distinct set of chemokines when re-challenged with a higher LPS concentration<sup>662</sup>. This group showed a differential desensitising effect with some factors remaining permanently suppressed (MIP-1a, MIP-1b, RANTES, TNF-a and IL-6) while other re-challenged monocytes were still able to produce the chemokines IL-8, GROa and MCP-1. Alternative activation of macrophages has been described (reviewed in<sup>287,352,663</sup>). Whether such effects, together with crosstalk between TLRs allows for further polarisation of the innate response, remains to be seen.

#### **5.9.4 Differentiating effects of Shh from those of endotoxic activity**

In light of the above discussion, is there a good way of differentiating between effects attributable to the protein of interest from those due to contaminating

endotoxin when both are proposed to signal through the same pathways? In the work presented here, the initial approach used was to quantify the endotoxic activity present in the rShh and then compare this directly with a dose of LPS of equivalent endotoxic activity. Clearly, although rShh apparently induced a greater magnitude of change than that induced by an equivalent concentration of LPS, in light of the subsequent data, this process alone was inadequate for differentiating effects related to the protein of interest from those due to endotoxin contamination. Some of the reasons why this process was inadequate relate to the possible nature of the bacterial components present within either the rShh or the LPS used as a control and have been discussed earlier.

Complicating matters further, there are a number of inherent difficulties associated with the accurate quantification of endotoxic activity particularly at low concentrations. These have been highlighted by a number of authors<sup>604,605,617,664</sup>. Of note is the fact that, as a bioassay, the LAL assay is much less precise than an immunologically based test such as an ELISA. Quantitation of the LAL enzyme activity requires optimal conditions and measurement may be complicated by a number of interference factors such as coloured or turbid solutions. Also, both endotoxin and LAL reagent are subject to biological variation<sup>665</sup> and the intra-test coefficient of variability of the LAL assay is not insignificant, previously reported to be in the order of ~50%<sup>604</sup>. Consequently, in many respects, the assay is only semi-quantitative with a value of, for example 4.5pg/ml LPS, not being significantly different, in terms of endotoxic activity, from 7pg/ml LPS. Thus, this further compounds the difficulties outlined above in attempts to extrapolate relative potencies of rShh solutions from LPS curves. Furthermore, endotoxins exhibit a significant ability to interact with other proteins and lipids including other bacterial components and components of serum<sup>666-671</sup> and such interactions can readily mask endotoxin molecules from detection and removal techniques<sup>672-674</sup>. In addition, the close association of the protein of interest with endotoxin, as likely to be the case in recombinant proteins, means that the endotoxic activity present within the sample may be significantly underestimated<sup>672,675,676</sup>. Serum, albumin, plasma and medium also can interfere with p-nitroaniline-based chromogenic assays because of their

yellow colour and turbidity<sup>602</sup> and it is thus perhaps not surprising that apparently higher results were obtained for IMDM and X-vivo 10 in the endpoint assay compared with results obtained from the thermokinetic assay.

Differentiating effects attributable to a protein of interest from endotoxin effects has received considerable recent attention in the field of TLR biology. As noted previously, a wide range of structurally and functionally diverse agonists have been proposed for TLR4, more so than for any other TLR (Reviewed in <sup>291,305,573</sup>). Given that the majority of these studies have used bacterially-derived or recombinant proteins, one longstanding concern has been that the effects seen may be attributable to endotoxin contamination of the proteins used. Indeed, recent data adds to these concerns with convincing evidence suggesting that cytokine effects attributed to rHSP60 and rHSP70 are caused by endotoxin contaminants<sup>595,609,610</sup>. Commonly employed techniques to differentiate between effects related to the protein of interest from those of endotoxin have included the differential sensitivity of the protein and endotoxin to the LPS neutralising agent PMB. However, although PMB is an effective neutraliser of several types of LPS (and, indeed, was particularly successful in its action in the work presented here), it is not capable of neutralising all forms of LPS<sup>613,677,678</sup>. Furthermore, in some instances, it has been reported to stimulate cytokine and complement production<sup>679,680</sup>, especially at high concentrations (~100µg/ml) and may even synergise with LPS<sup>613</sup>. A further consideration is that the lipoprotein and/or phospholipid components of endotoxins and commercial LPS preparations are not reliably inhibited by PMB<sup>634,681</sup>. For these reasons, PMB may not always be a suitable method for differentiating between protein-related and endotoxin-related effects. The differential sensitivity of proteins and LPS to heat is another method commonly employed in the literature to differentiate between a protein of interest and endotoxin. Classically, LPS has been reported to be heat resistant<sup>322-324,575</sup>. However, recently, Gao and Tsan have suggested that, depending on the concentration of LPS present, it may be very heat sensitive<sup>609,610</sup>. They posited that if 99% of LPS was inactivated by heat, then the significance of this would be different depending on the starting concentration of LPS present. The work presented



in this chapter is in keeping with the reports of Gao and Tsan, finding that both 2000ng/ml rShh and 100pg/ml LPS are, indeed, heat-sensitive.

Thus, from the above it can be seen that differentiating protein-related effects from those of contaminants in bacterially-derived recombinant proteins is difficult. The experiments outlined in this chapter would be aided considerably if pure Shh ligand could be used. Currently such a protein is not commercially available. It may be possible to generate Shh protein in a non-bacterial expression system such as Chinese hamster ovary (CHO) cells, which, although not eliminating the effects of possible exogenous endotoxin contamination, has the potential to result in the production of a purer protein. Alternatively, with the development of better antibodies to Shh, it may be possible to extract Shh from tissues, although such a process is complicated by the relatively low expression of Shh in adult tissues. Under this circumstance, use of embryonic tissue such as murine brain may prove a more viable alternative. The production of a pure preparation of Shh would dramatically aid our understanding of how macrophages may respond to Shh and, indeed, whether Shh has any effect on macrophage cytokine and chemokine effector function.

### 5.9.5 Summary

The work presented in this chapter describes the expression of Shh signalling pathway components in human PBMC-derived macrophages and, further, reports on the effect of recombinant Shh protein on the cytokine and chemokine effector function of these cells. The effects on cytokine and chemokine effector function are enhanced by serum, differentially modulated by the specific Shh signalling pathway inhibitor, cyclopamine, and consistently abrogated by anti-CD14 blockade. These results together with the dramatic inhibition of the response by the LPS neutralising agent PMB suggest that endotoxin contamination present within the E coli – derived recombinant Shh protein utilised to test the effect of Shh *in vitro*, is primarily responsible for the stimulatory effects seen. Further clarification of a possible role for Shh in macrophages requires that pure preparations of Shh are available for future investigation.

**Chapter 6**

**The Effect of Sonic Hedgehog on T cell Effector  
Function**

## 6.1 Introduction

A growing body of evidence suggests that Hh signalling may play a role in the developing and mature immune system. Outram et al<sup>147</sup> demonstrated that Shh signalling is present during murine thymocyte development and that it regulates the development of double positive from double negative thymocytes. Subsequently, work by Bhardwaj et al<sup>19</sup> observed that Shh signalling is important in the proliferation of haematopoietic stem cells. More recent data from our group has suggested that Shh signalling may play a role in the adult immune system. Most notably, adult murine and human CD4<sup>+</sup> T cells have been shown to express transcripts for *Shh* and *Ptc* and, that these cells can produce and respond to a Hh signal, has been demonstrated in 2 studies<sup>20,21</sup>. Lowrey et al<sup>21</sup> observed that the addition of rShh protein to cultures of murine CD4<sup>+</sup> T cells, sub-optimally activated using anti-CD3 and anti-CD28 antibodies, enhanced T cell proliferation, an effect subsequently shown to occur through the enhanced entry into the S/G2 proliferative phase of the cell cycle. Stewart et al<sup>20</sup> extended this work further, investigating Shh signalling in human CD4<sup>+</sup> T cells. The results were broadly in agreement with the earlier findings of Lowrey et al<sup>21</sup>. Notably, the proliferation of activated CD4<sup>+</sup> T cells was enhanced by exogenous Shh in approximately 50% of human donors studied, an effect accompanied by a modulation in cytokine effector function.

The reports outlined above present a number of interesting avenues for further enquiry. For example, although Hh signalling has been shown to modulate the proliferation and cytokine effector function of CD4<sup>+</sup> T cells *in vitro* using anti-CD3 and anti-CD28 antibodies, it is not known whether, if antigen presenting cells are substituted for these activation signals, the same effect on CD4<sup>+</sup> T cell effector function will occur. Furthermore, given that Shh is upregulated at sites of epithelial damage<sup>149</sup>, that rShh enhances the proliferation of activated CD4<sup>+</sup> T cells<sup>20,21</sup> and that macrophages express *Ptc*<sup>149</sup>, it is conceivable that Shh signalling may be involved in or fuel interactions occurring at the epithelial-immune cell interface during inflammation or repair.

## **6.2 Shh signalling and the effector function of depletion-purified, anti-CD3 $\epsilon$ /anti-CD28 activated, CD4+ T cells**

In light of the previous observations that rShh enhances the proliferation and cytokine effector function of activated, but not resting, murine and human CD4+ T cells<sup>20,21</sup>, the aim of this section was to repeat the experiments previously reported by Stewart et al<sup>20</sup> determining the effect of rShh on human CD4+ T cells. In these experiments, exogenous rShh was shown to enhance the proliferation of depletion-purified CD4+ T cells in 6 of 13 donors<sup>20</sup>. Also, in those donors where proliferation was enhanced, the cytokine production by these cells was also modulated by exogenous rShh administration.

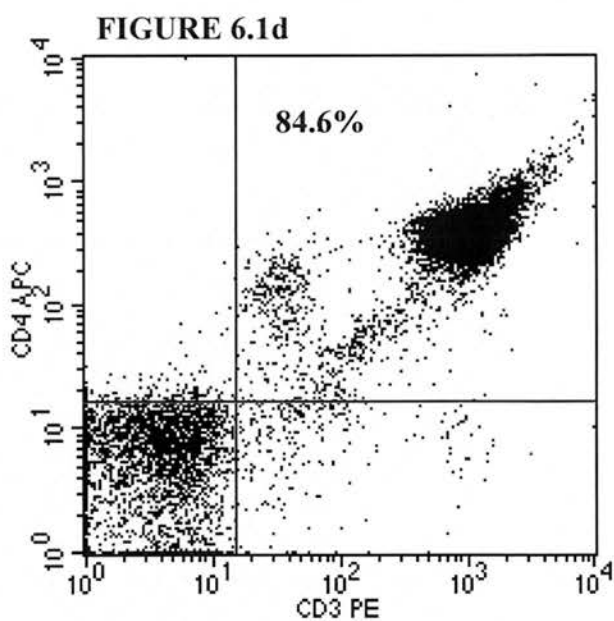
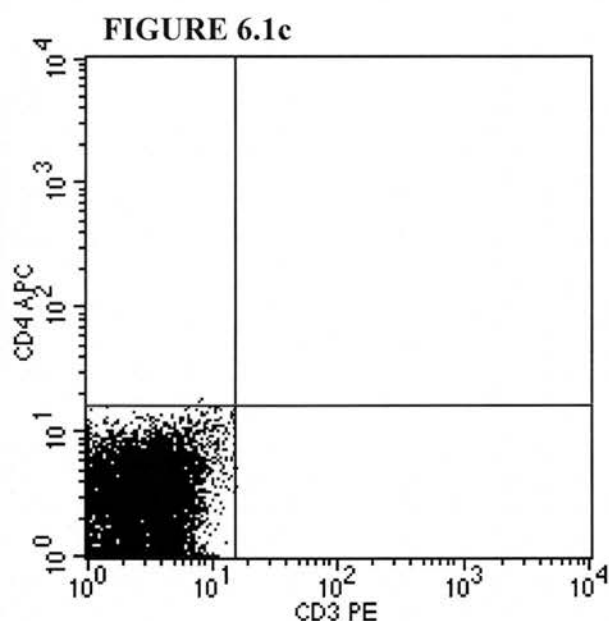
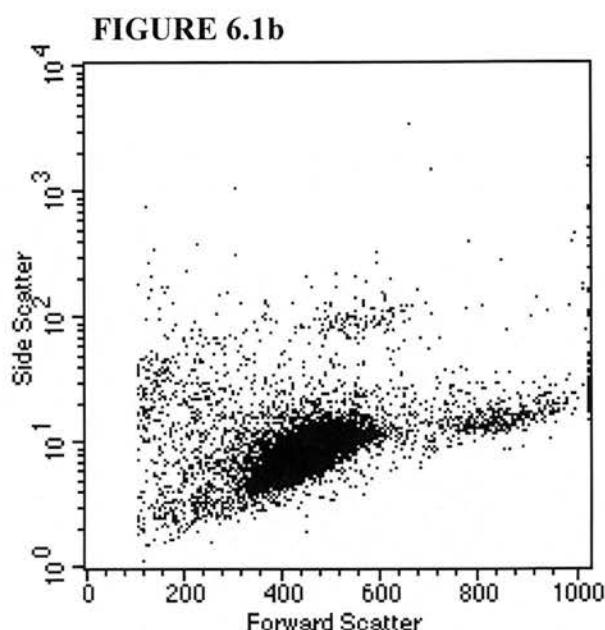
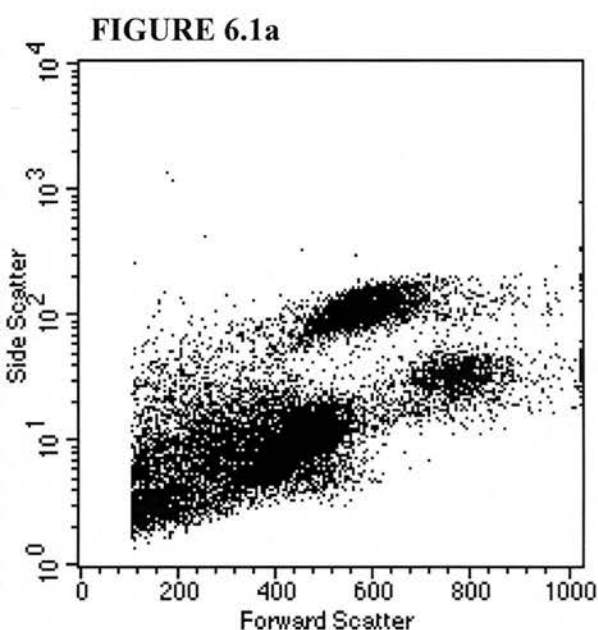
Therefore, the aim of this section was to see whether I could reproduce these results using rShh and human peripheral blood mononuclear cell (PBMC)-derived CD4+ T cells.

### **6.2.1 Isolation and depletion purification of human CD4+ T cells**

As described in Chapter 2, PBMCs were isolated from normal, single donor Buffy coats obtained from Scottish National Blood Transfusion Service, Edinburgh. Each Buffy coat was made up to 200mls using PBS and PBMCs were obtained by centrifugation over Lymphoprep (Gibco) for 25 minutes at 1000g. The PBMCs, appearing at the interface between the Lymphoprep and serum were removed and washed twice in PBS to remove platelets. The PBMCs were reconstituted in serum-free RPMI and plated into culture flasks at  $5 \times 10^6$ /ml. The cells were incubated for 1 hour at 37°C to allow adherent monocytes to be depleted from the PBMCs, by adherence to the plastic culture flask. Non-adherent cells were removed, counted and washed in PBS prior to proceeding to cell separation.

As previously<sup>20</sup>, CD4<sup>+</sup> T cells were isolated from the PBMCs using CD4<sup>+</sup> depletion columns (R&D Systems) according to the manufacturer's instructions. Briefly,  $20 \times 10^7$  PBMCs were reconstituted in 1 x column buffer (R&D Systems) and then mixed with the antibody cocktail (R&D Systems). B cells and other non-CD4 cells bind to anti-Ig coated glass beads via Fc interactions, leaving the CD4<sup>+</sup> T cells unbound. Following washing, the cell-bead mix was transferred to the separation column (R&D Systems) and the cells were incubated on the column for 10 minutes at room temperature. CD4<sup>+</sup> T cells were eluted from the column using 10mls of column buffer, leaving bound cells (and, thus, non-CD4<sup>+</sup> T cells) retained on the column. The cells were then counted, washed and reconstituted into RPMI containing 10% (heat inactivated) human AB serum (Sigma).

Following this isolation, purities of CD4<sup>+</sup> T cells in the order of 85% (range 82 – 88%) (Figure 6.1) were obtained as determined by flow cytometry employing double-staining techniques using Phycoerythrin (PE)-labelled, mouse anti-human CD3 and Allophycocyanin (APC)-labelled, mouse anti-human CD4 antibodies (BD Pharmingen, San Diego, CA, USA).



**FIGURE 6.1**

**Representative flow cytometry data demonstrating purities of CD4+ T cells isolated using depletion techniques**

Following centrifugation over Lymphoprep, CD4+ T cells were isolated from PBMCs using CD4 depletion columns (R&D Systems). Representative flow cytometry scatter plots for non-adherent PBMCs (Figure 6.1a), unstained depletion-purified CD4+ T cells (Figure 6.1b) and the isotype controls (Figure 6.1c) are shown. Purities of CD3+CD4+ T cells were confirmed using PE-labelled and APC-labelled mouse anti-human antibodies to CD3 and CD4 respectively (Figure 6.1d). Depletion-purified CD4+ T cells yielded purities in the range 82-88%.



## 6.2.2 Shh and CD4<sup>+</sup> T cell proliferation

In this series of experiments, depleted CD4<sup>+</sup> T cells were prepared as outlined in 6.2.1. As described previously<sup>20,21</sup>, the CD4<sup>+</sup> T cells were activated using monoclonal antibodies to anti-CD3 and anti-CD28 (BD Pharmingen). Prior to cell culture experiments, the anti-CD3 $\epsilon$  antibody (BD Pharmingen) was immobilised onto the wells of 96-well plates by diluting the antibody in PBS to final concentrations of either 0.2 or 1  $\mu$ g/ml as previously<sup>20,21</sup>. The plates were incubated for 2 hours at 37°C and then washed 3 times with PBS to remove unbound antibody. The plates were air-dried for 30 minutes at 37°C prior to use. Depleted CD4<sup>+</sup> T cells were incubated at  $2 \times 10^5$  cells per well either in medium alone (resting T cells) or in the presence of plate-bound anti-CD3 $\epsilon$  and soluble anti-CD28 antibodies (activated T cells). Each condition was replicated in quadruplicate on the 96-well plate. Anti-CD3 $\epsilon$  at 0.2  $\mu$ g/ml with 1  $\mu$ g/ml anti-CD28 constituted a “suboptimal” activation stimulus and anti-CD3 $\epsilon$  at 1  $\mu$ g/ml and anti-CD28 at 5  $\mu$ g/ml constituted an “optimal” activation stimulus in keeping with previous studies<sup>20,21</sup>. Parallel experiments were performed in which 10-1000 ng/ml rShh was added to these cultures at the initiation of culture.

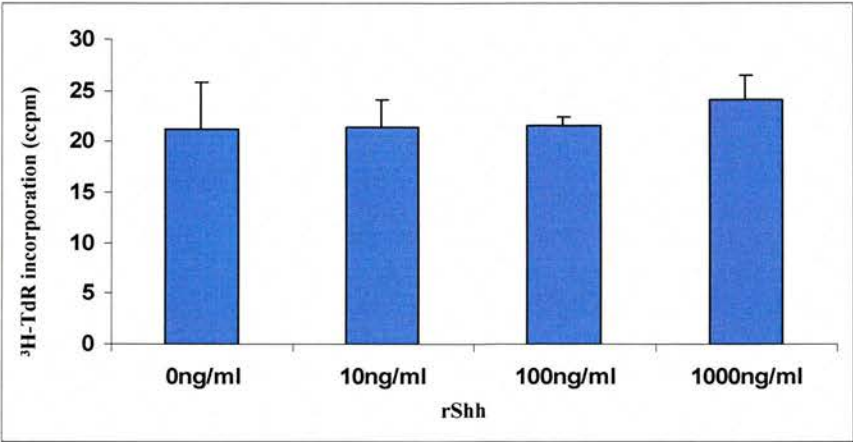
After 72 hours in culture, 20  $\mu$ l of cell culture supernatant was removed from each well (thus providing 80  $\mu$ l for each culture condition) and set aside for later cytokine analysis. To evaluate proliferation, the cells were pulsed with tritiated methyl thymidine (1  $\mu$ Ci/well) (<sup>3</sup>H-TdR-Amersham Pharmacia Biotech, Amersham, UK) and incubated for a further 18 hours. <sup>3</sup>H-TdR incorporation was evaluated using a Betaplate 1205 liquid scintillation counter (Wallac, Milton Keynes, UK). The experiment was performed in 12 donors.

Cytokine analysis on the supernatants was performed using the Th1/Th2 cytometric bead array (CBA) (BD Biosciences), as described in Chapter 2. Using the Th1/Th2 CBA, the following cytokines can be detected: Interferon-gamma (IFN $\gamma$ ), Tumour Necrosis Factor-alpha (TNF $\alpha$ ) and interleukins IL-10, IL-6, IL-4 and IL-2.

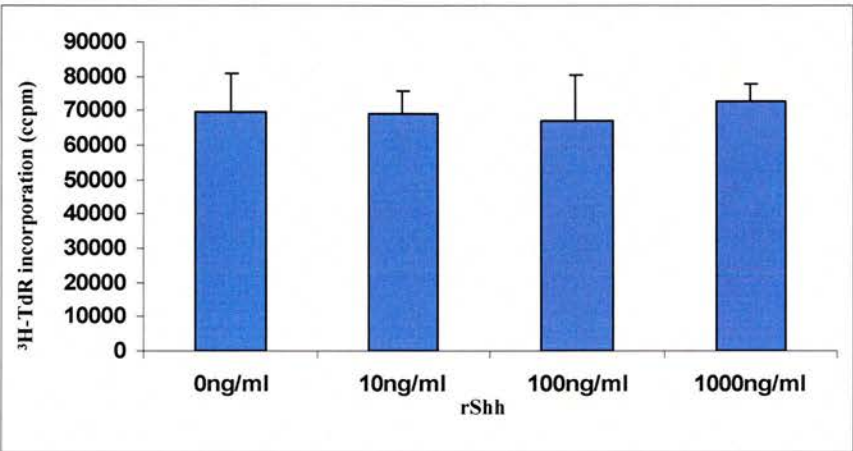
The results are shown in Figure 6.2. Consistent with previous reports<sup>20,21</sup>, rShh failed to modulate the proliferation of unstimulated CD4<sup>+</sup> T cells (Figure 6.2a). However, in contrast to the previous studies, enhanced proliferation in response to rShh in activated CD4<sup>+</sup> T cells was not observed across any of the concentrations of rShh used. Furthermore, this failure to modulate the proliferation of CD4<sup>+</sup> T cells was observed, irrespective of whether sub-optimal (Figure 6.2b) or optimal (Figure 6.2c) activation conditions were employed.

Figure 6.3 and Figure 6.4 demonstrate the effect of rShh on cytokine release from suboptimally (Figure 6.3) and optimally (Figure 6.4) activated CD4<sup>+</sup> T cells at 72 hours. For the purposes of statistical analysis, rShh-treated CD4<sup>+</sup> T cells were compared to untreated controls using a two-tailed t-test for paired samples. A p value of <0.05 was considered significant (\* p<0.05, \*\* p<0.01, \*\*\* p<0.001). As shown, the addition of rShh upregulated significantly the production of IFN- $\gamma$ , TNF $\alpha$ , IL-10, IL-6 and IL-2, but not IL-4, in suboptimally activated CD4<sup>+</sup> T cells. Conversely, in optimally activated CD4<sup>+</sup> T cells, the addition of rShh failed to modulate significantly the cytokine effector function of CD4<sup>+</sup> T cells (Figure 6.4).

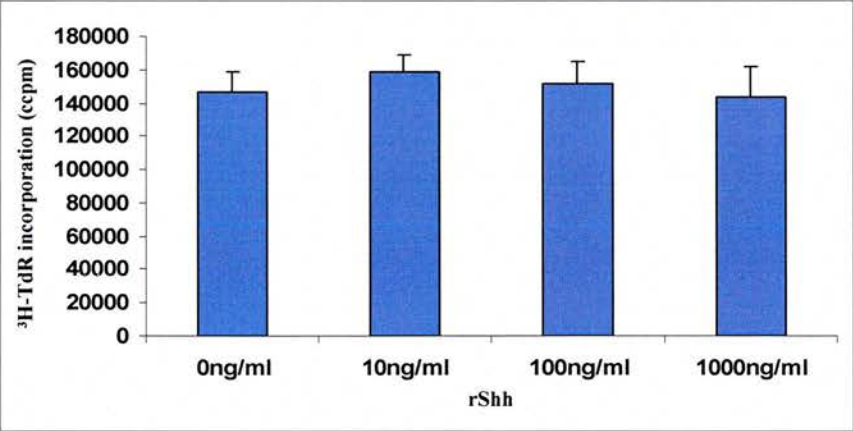
**FIGURE 6.2a**



**FIGURE 6.2b**



**FIGURE 6.2c**



**FIGURE 6.2**  
**rShh fails to modulate the proliferation of resting or activated depletion-purified CD4+ T cells**

Resting (Figure 6.2a) or anti-CD3 $\epsilon$ /anti-CD28 activated CD4+ T cells were cultured in the presence or absence of 10-1000ng/ml rShh for 72 hours. Proliferation was assayed by <sup>3</sup>H-TdR incorporation over 18 hours. Irrespective of whether the cells were suboptimally (0.2  $\mu$ g/ml anti-CD3 $\epsilon$ : 1 $\mu$ g/ml anti-CD28) (Figure 6.2b) or optimally (1 $\mu$ g/ml anti-CD3 $\epsilon$ : 5 $\mu$ g/ml anti-CD28)(Figure 6.2c) activated, rShh failed to modulate the proliferation of the activated T cells. The experiment was performed in 12 individual donors. Results are representative of at least 5 experiments and means and standard deviations are shown.

Figure 6.3a

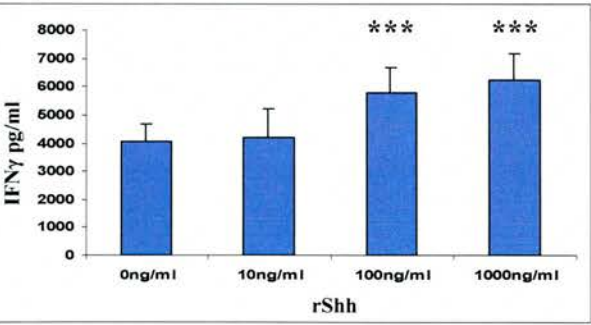


Figure 6.3b

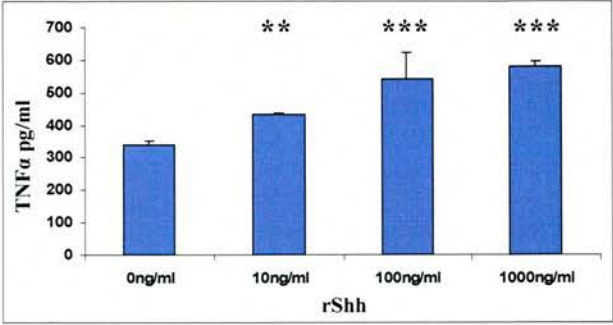


Figure 6.3c

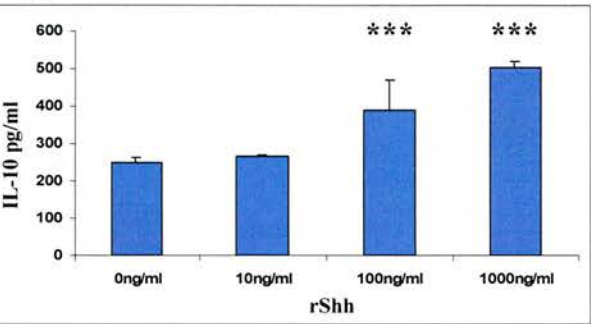


Figure 6.3d

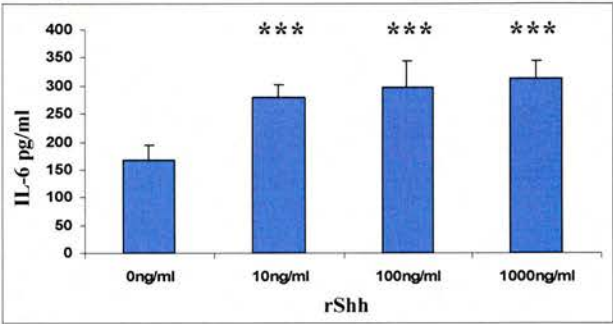


Figure 6.3e

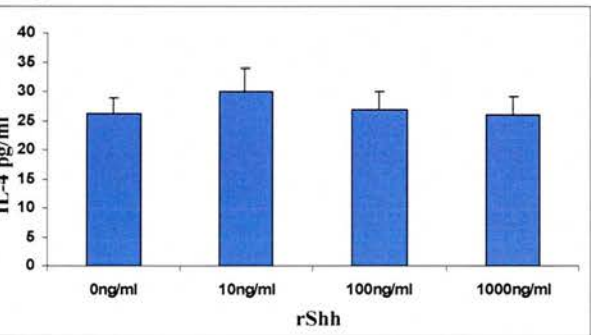


Figure 6.3f

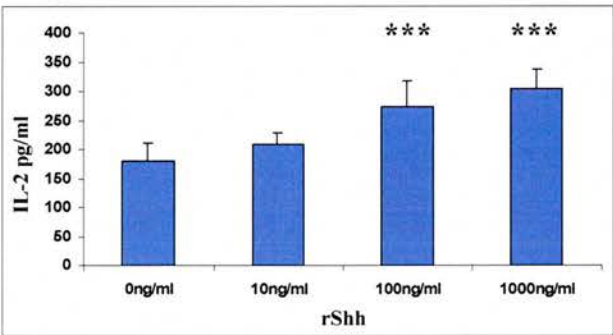


FIGURE 6.3

**rShh upregulates the production of IFN $\gamma$ , TNF $\alpha$ , IL-10, IL-6 and IL-2 from suboptimally activated CD4 $^{+}$  T cells**

Depletion-purified CD4 $^{+}$  T cells were suboptimally activated using monoclonal anti-CD3 $\epsilon$  and anti-CD28 antibodies at 0.2 $\mu$ g/ml and 1 $\mu$ g/ml respectively for 72 hours in the presence and absence of 10-1000ng/ml rShh. Supernatants were analysed for cytokine production using cytometric bead arrays. rShh upregulated the production of IFN $\gamma$  (a), TNF $\alpha$  (b), IL-10 (c), IL-6 (d) and IL-2 (f). Conversely, IL-4 was not modulated by rShh (e) For the purposes of statistical analysis, treated CD4 T cells were compared to untreated controls using a two-tailed paired t-test. Results shown are representative of at least 5 donor experiments and means and standard deviations are shown.

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$



Figure 6.4a

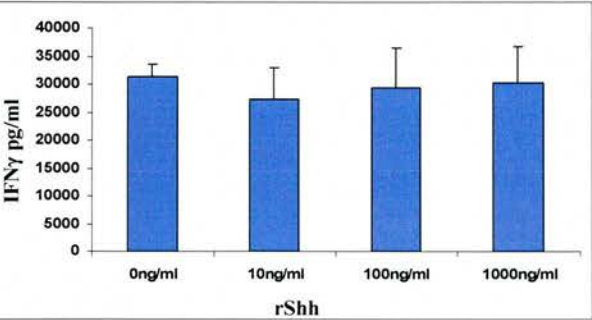


Figure 6.4b

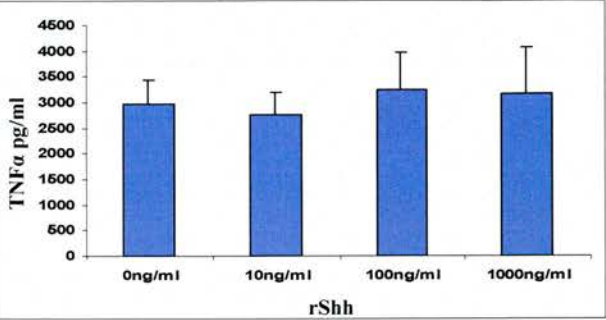


Figure 6.4c

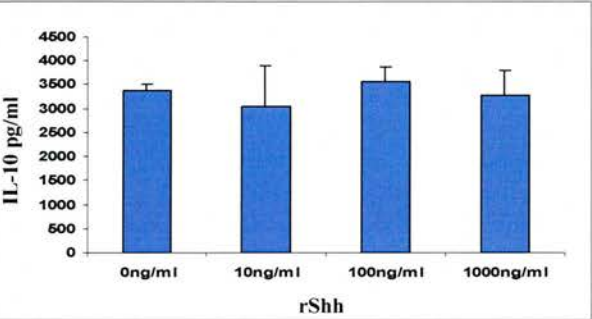


Figure 6.4d

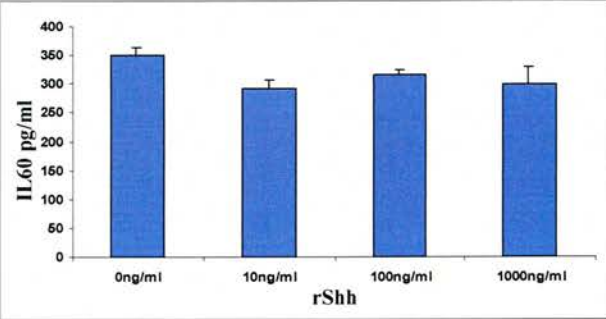


Figure 6.4e

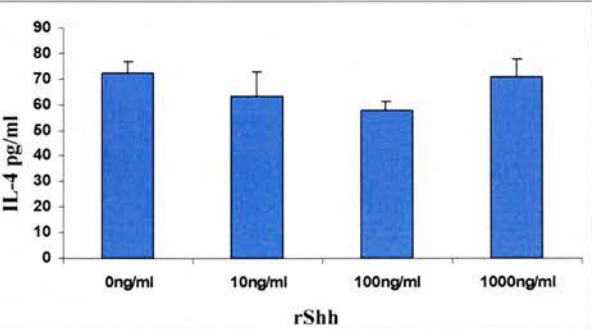
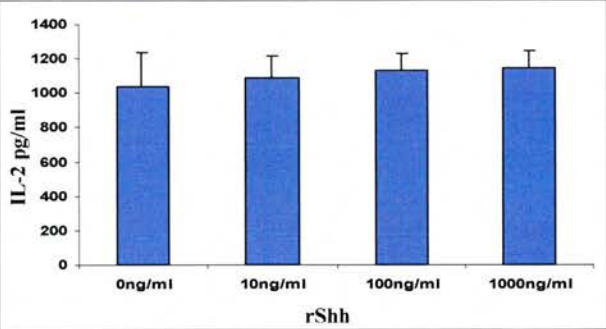


Figure 6.4f



**FIGURE 6.4**  
**rShh fails to modulate the production of IFN $\gamma$ , TNF $\alpha$ , IL-10, IL-6, IL-4 and IL-2 from optimally activated CD4+ T cells**

Depletion-purified CD4+ T cells were optimally activated using monoclonal anti-CD3 $\epsilon$  and anti-CD28 antibodies at 1 $\mu$ g/ml and 5 $\mu$ g/ml respectively for 72 hours in the presence and absence of 10-1000ng/ml rShh. Supernatants were analysed for cytokine production using cytometric bead arrays. rShh failed to modulate the production of IFN $\gamma$  (a), TNF $\alpha$  (b), IL-10 (c), IL-6 (d), IL-4 (e) and IL-2 (f). For the purposes of statistical analysis, treated CD4 T cells were compared to untreated controls using a two-tailed paired t-test. Results shown are representative of at least 5 donor experiments and means and standard deviations are shown.

### **6.3 Shh signalling and the effector function of depletion-purified CD4<sup>+</sup> T cells in the presence of an antigen presenting cell**

From the experiments outlined above, it was apparent that, although rShh failed to modulate the proliferation of depletion-purified CD4<sup>+</sup> T cells, rShh did appear to modulate the cytokine effector function of suboptimally activated CD4<sup>+</sup> T cells, a finding in keeping with those of Stewart et al<sup>20</sup>.

Thus, the aim of these experiments was to determine whether, if the anti-CD3 $\epsilon$ /anti-CD28 ligation was replaced by the presence of antigen presenting cells, the same effect of rShh on CD4<sup>+</sup> T cell effector function would be observed. For these experiments, given that macrophages express the receptor for Shh, Ptc, and, thus, have the potential to interact with CD4<sup>+</sup> T cells via the Shh signalling pathway, macrophages were selected as the antigen presenting cells of choice in this system. Monocytes were isolated from human PBMCs as described in Chapter 2 and adhered into macrophages over a period of 6 days as in Chapter 5. Following this incubation, the cells were treated with Mitomycin-C (MMC)(Sigma), an antibiotic that inhibits nucleic acid synthesis. Briefly, cells were mixed with 50 $\mu$ g/ml MMC and incubated for 30 minutes at 37°C. The cells were then thoroughly washed 4 times with ice-cold medium to remove any residual MMC. Cells prepared in this way were counted and plated out into round-bottomed 96 well culture plates at a range of concentrations from 10<sup>4</sup> - 5 x 10<sup>5</sup> cells per well.

Depletion-purified CD4<sup>+</sup> T cells from a third party donor were prepared as outlined in Section 6.2.1 above and added to the MMC-treated macrophages using 2 x 10<sup>5</sup> CD4<sup>+</sup> T cells per well in the presence and absence of 10-1000ng/ml rShh. As previously, each culture condition was performed in quadruplicate. The mixed lymphocyte reactions (MLRs) so prepared were left in culture for a period of 72 hours. At the end of this time, 20 $\mu$ l of cell culture supernatant was removed from each well and set aside for later cytokine analysis using the Th1/Th2 CBA as in



Section 6.2. The cells were pulsed with  $^3\text{H}$ -TdR (1 $\mu\text{Ci}$ /well) and incubated for a further 18 hours.  $^3\text{H}$ -TdR incorporation was evaluated as in Section 6.2. The experiment was performed in 8 T cell donors.

The results from these experiments are shown in Figures 6.5 and 6.6. As shown in Figure 6.5, irrespective of the number of MMC-treated macrophages in culture, rShh failed to modulate the proliferation of the MLRs at any of the concentrations of rShh employed. Figure 6.6 demonstrates the effect of rShh on the cytokines produced in the MLRs. As shown, the addition of rShh failed to modulate either the cytokine profile or the magnitude of the cytokine response seen and this was observed irrespective of the number of antigen presenting cells present or the concentration of rShh used.

FIGURE 6.5a

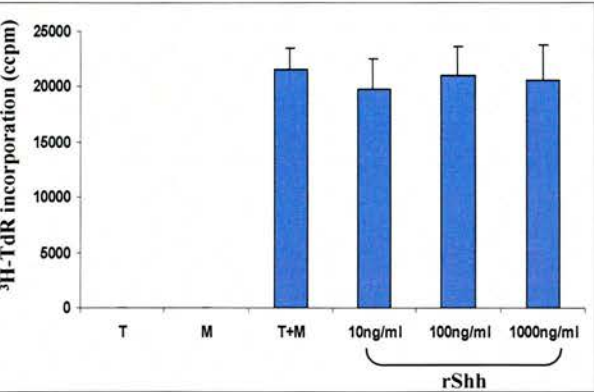


FIGURE 6.5b

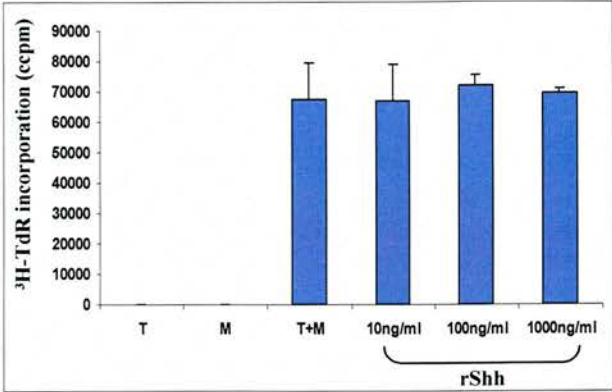


FIGURE 6.5c

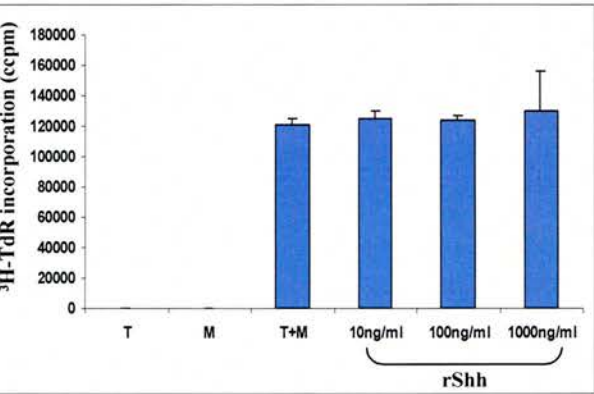


FIGURE 6.5d

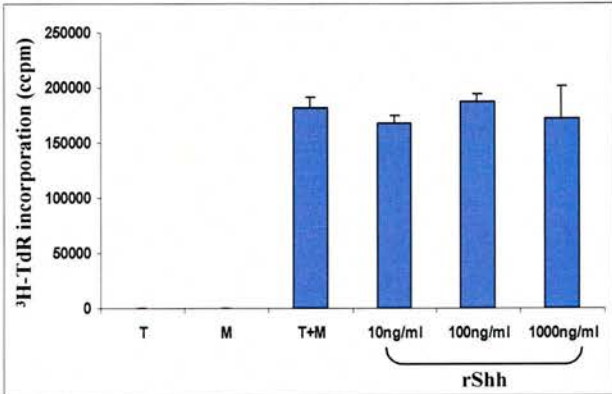


FIGURE 6.5

**rShh fails to modulate the proliferation of T cells in mixed leukocyte reactions (MLRs)**

Depletion-purified CD4+ T cells were added to cultures of mitomycin C (MMC)-treated macrophages. 10<sup>4</sup> (Figure 6.5a), 5x10<sup>4</sup> (Figure 6.5b), 10<sup>5</sup> (Figure 6.5c) and 5x10<sup>5</sup> (Figure 6.5d) MMC-treated macrophages were used to stimulate the CD4+ T cells. For each set of culture conditions, T cells alone (T) and MMC-treated macrophages alone (M) were run alongside the MLR reaction (T+M) and in the presence of 10-1000ng/ml rShh. Irrespective of the number of MMC-treated macrophages used as stimulator cells, rShh failed to modulate the proliferative response of the CD4+ T cells. The experiment was performed in 8 T cell donors. Results are representative of at least 4 experiments and means and standard deviations are shown.

Figure 6.6a

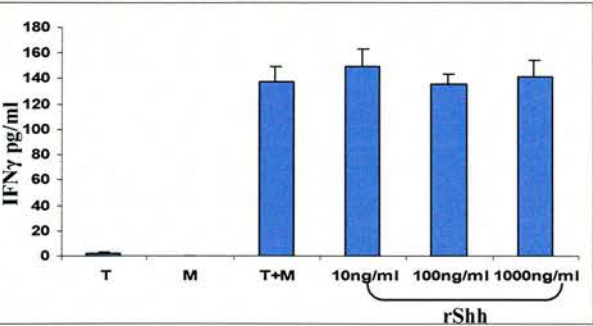


Figure 6.6b

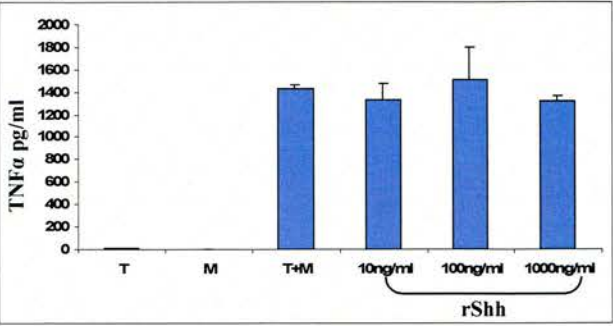


Figure 6.6c

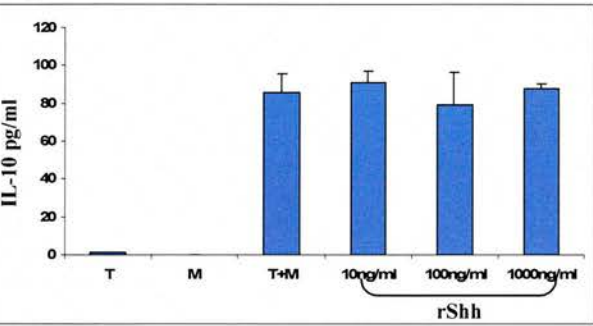


Figure 6.6d

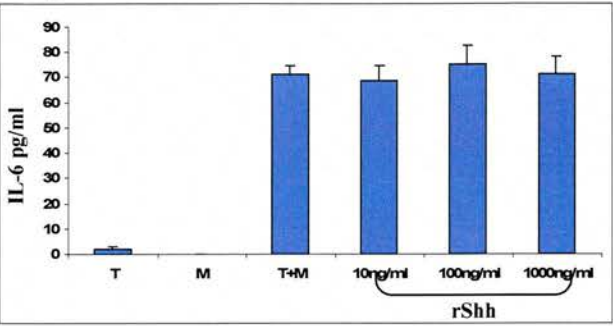


Figure 6.6e

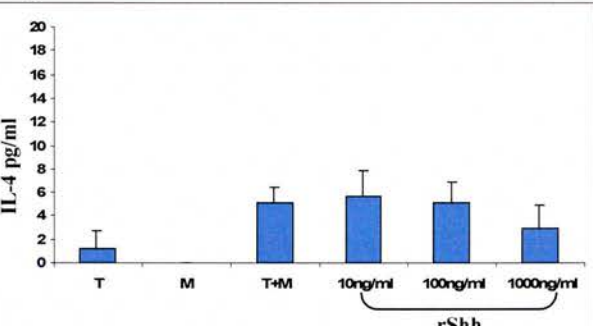


Figure 6.6f

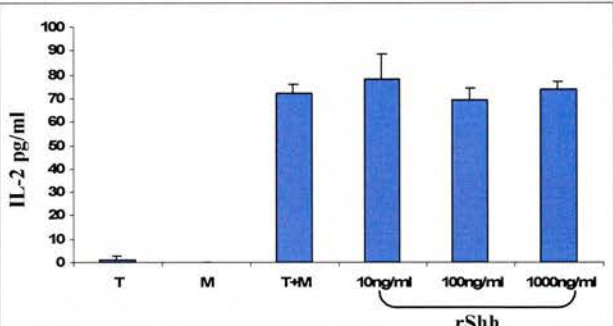


FIGURE 6.6

**rShh fails to modulate the cytokine effector function of T cells in MLRs**

Depletion-purified CD4<sup>+</sup> T cells were added to cultures of mitomycin C (MMC)-treated macrophages in the concentration range  $10^4$  -  $5 \times 10^5$  per well. 72 hour culture supernatants were analysed for cytokine production using cytometric bead arrays. For each set of culture conditions, T cells alone (T) and MMC-treated macrophages alone (M) were run alongside the MLR reaction (T+M) and in the presence of 10-1000ng/ml rShh. The results using  $5 \times 10^4$  MMC-treated macrophages are shown. rShh failed to modulate the proliferative response of the CD4<sup>+</sup> T cells, irrespective of the number of stimulator macrophages used. The experiment was performed in 8 T cell donors. Results are representative of at least 4 experiments and means and standard deviations are shown.

## **6.4 The effect of Shh blockade on T cell effector function**

CD4<sup>+</sup> T cells have been demonstrated to express Shh at both the mRNA and protein level<sup>20,21</sup>. Furthermore, it has been shown that, in anti-CD3 $\epsilon$ /anti-CD28 activated, depletion-purified CD4<sup>+</sup> T cells, blockade of Shh signalling, using the monoclonal antibody, 5E1, inhibits the proliferation of CD4<sup>+</sup> T cells<sup>20,21</sup>. In the C57BL/6J mouse, this effect was consistent across all experiments<sup>21</sup>. Conversely, in human CD4<sup>+</sup> T cells, Stewart et al<sup>20</sup> observed inhibition of proliferation in only 50% donors. In those ‘responding’ donors, the upregulation of IL-2 and IFN- $\gamma$ , but not IL-5 and IL-10, could also be abrogated by 5E1<sup>20</sup>. The aim of this series of experiments was to determine the effect of Shh signalling blockade on T cell function.

### **6.4.1 Blocking Shh signalling in anti-CD3/anti-CD28 activated, depletion-purified CD4<sup>+</sup> T cells fails to modulate T cell effector function**

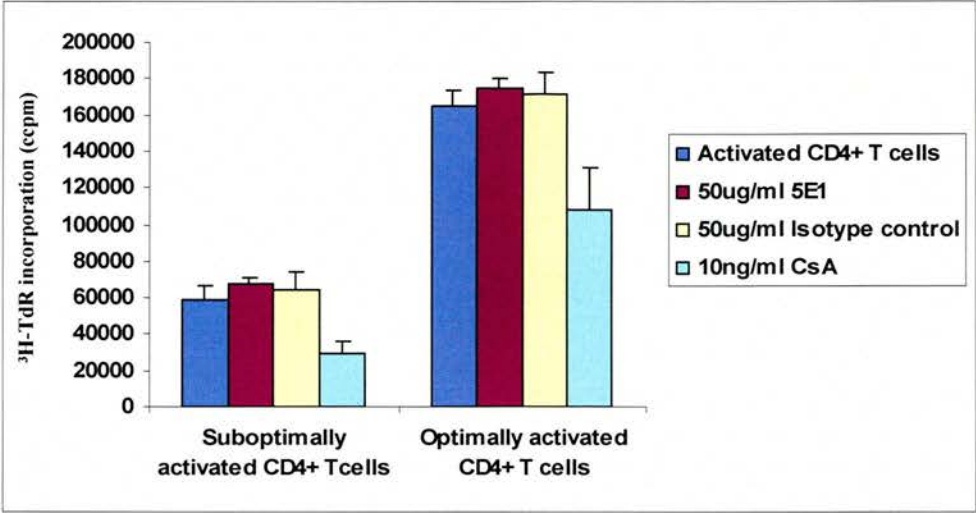
In light of the previous findings of Stewart et al<sup>20</sup> and Lowrey et al<sup>21</sup>, suggesting that 5E1 could abrogate the proliferation and cytokine effector function, the aim of these experiments was to determine whether these results could be reproduced in my experiments using depletion-purified CD4<sup>+</sup> T cells. As previously, the mouse monoclonal antibody, 5E1, was employed. This antibody is thought to bind to Shh at a site distinct from, but possibly overlapping, the Ptc binding site<sup>654</sup>. Thus, it might be postulated that the addition of 5E1 to the cultures prevents endogenous CD4<sup>+</sup> T cell-derived Shh from activating the Shh signalling pathway in CD4<sup>+</sup> T cells.

As outlined previously in this chapter, human CD4<sup>+</sup> T cells were purified from PBMCs using negative selection affinity columns (R&D Systems). These cells were plated at  $2 \times 10^5$  cells per well of a 96 well plate in the presence of sub-optimal and optimal activation using anti-CD3 $\epsilon$  and anti-CD28 antibodies as in Section 6.2.2. At the initiation of culture, 5-50 $\mu$ g/ml 5E1 antibody was added to the wells, in keeping with previous studies<sup>19-21</sup>. Mouse IgG used at 50 $\mu$ g/ml served as the isotype control

as previously<sup>20,21</sup>. By way of a positive control, 10 ng/ml ciclosporin was added to the cultures in place of the 5E1 antibody.

The results are shown in Figures 6.7 and 6.8. Although ciclosporin successfully abrogated the proliferation of suboptimally and optimally activated CD4+ T cells, proliferation of these cells failed to be modulated by the addition of 5E1 (Figure 6.7). Furthermore, 5E1 failed to abrogate the cytokine effector function of suboptimally and optimally activated CD4+ T cells (Figure 6.8).

**FIGURE 6.7**



**FIGURE 6.7**

**The proliferation of suboptimally and optimally activated depletion-purified CD4+ T cells is not modulated by inhibition of Shh signalling**

Depletion-purified CD4+ T cells were suboptimally or optimally activated using anti-CD3 $\epsilon$  and anti-CD28 antibodies, in the presence or absence of 5-50ug/ml 5E1. 50ug/ml mouse IgG served as a negative control. 10ng/ml ciclosporin (CsA) was used as a positive control. Irrespective of whether the CD4+ T cells were suboptimally or optimally activated, ciclosporin abrogated T cell proliferation. Conversely, 5E1 failed to modulate the proliferation of activated T cells, irrespective of the concentration of 5E1 used. The results obtained using 50ug/ml 5E1 are shown. Results are representative of at least 4 experiments and means and standard deviations are shown.



Figure 6.8a

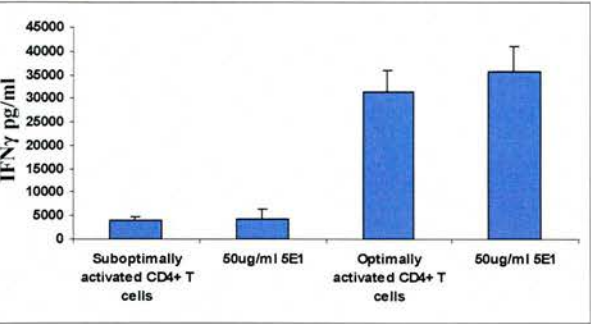


Figure 6.8b

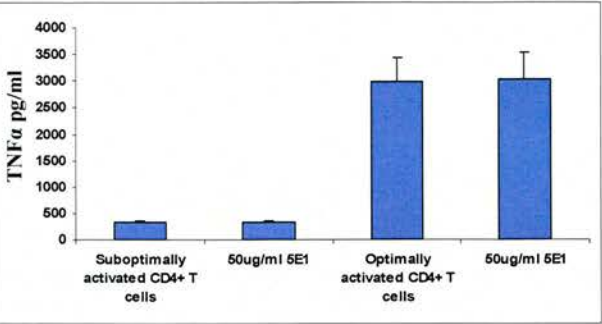


Figure 6.8c

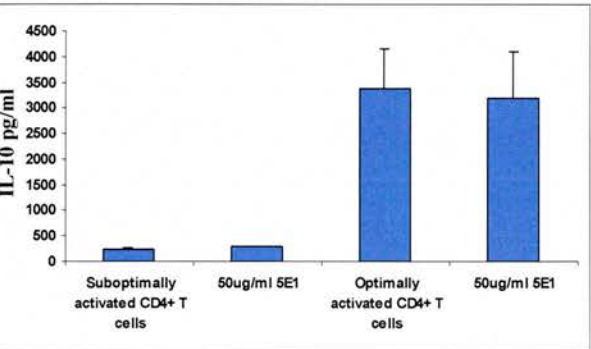


Figure 6.8d

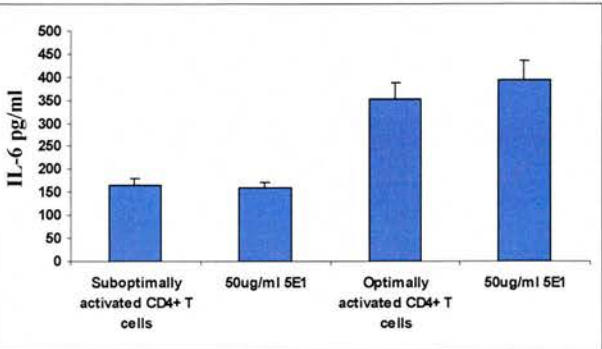


Figure 6.8e

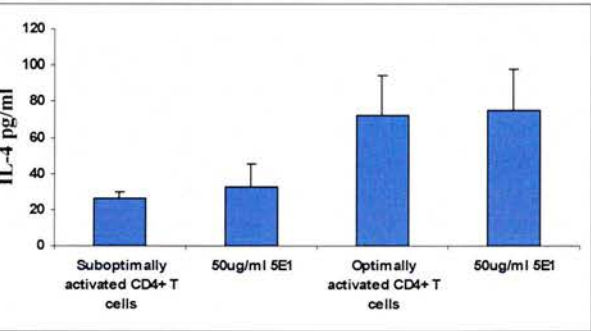


Figure 6.8f

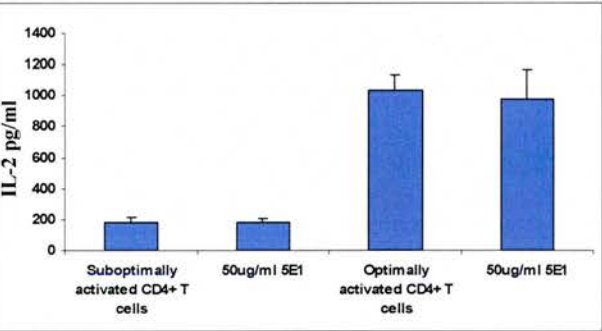


FIGURE 6.8

**The cytokine effector function of suboptimally and optimally activated depletion-purified CD4 $^{+}$  T cells is not modulated by inhibition of Shh signalling**

Depletion-purified CD4 $^{+}$  T cells were suboptimally or optimally activated using anti-CD3 $\epsilon$  and anti-CD28 antibodies, in the presence or absence of 5-50ug/ml 5E1. 5E1 failed to modulate the cytokine effector function of activated T cells, irrespective of the concentration of 5E1 used. The results obtained using 50ug/ml 5E1 are shown. Results are representative of at least 4 experiments and means and standard deviations are shown.

#### **6.4.2 Blocking Shh signalling in MLR cultures fails to modulate T cell effector function**

In parallel experiments to those outlined in Section 6.4.1 above, MLRs were prepared as described in Section 6.3. Briefly,  $10^4 - 5 \times 10^5$  macrophages were treated with MMC and used to stimulate  $2 \times 10^5$  third party, depletion-purified CD4<sup>+</sup> T cells. At the initiation of culture, 5E1 or 10 ng/ml ciclosporin were added to the cultures. Proliferation following 72 hours in culture was evaluated and 20 µl of supernatant per well was set aside for later cytokine analysis.

The results are shown in Figures 6.9 and 6.10. Ciclosporin abrogated the MLR response. Conversely, 5E1 failed to modulate the proliferation or cytokine profiles of the MLR.

FIGURE 6.9

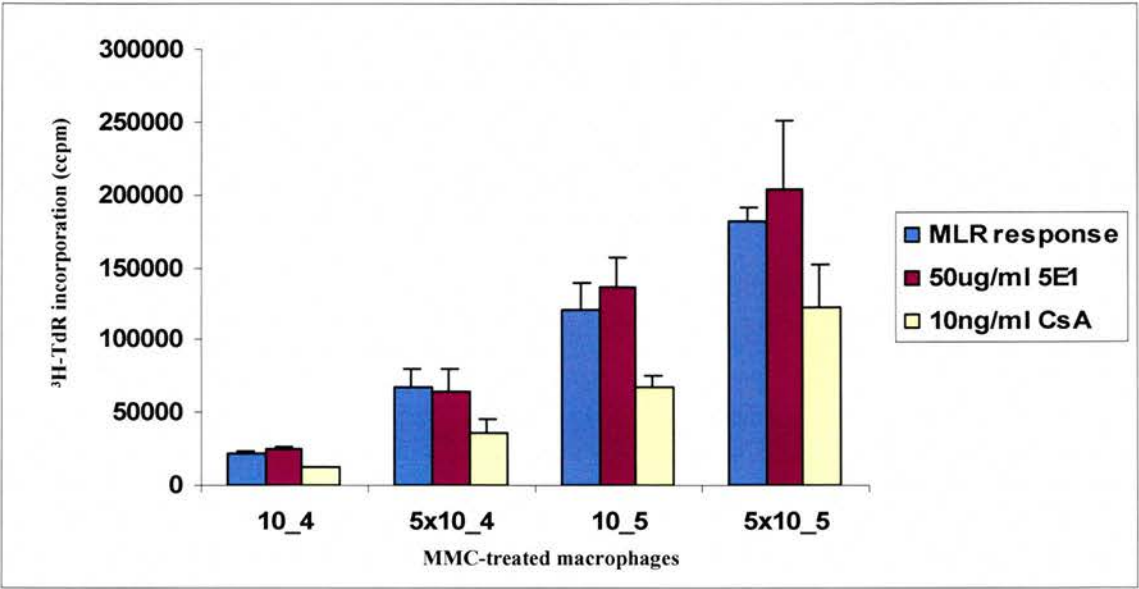
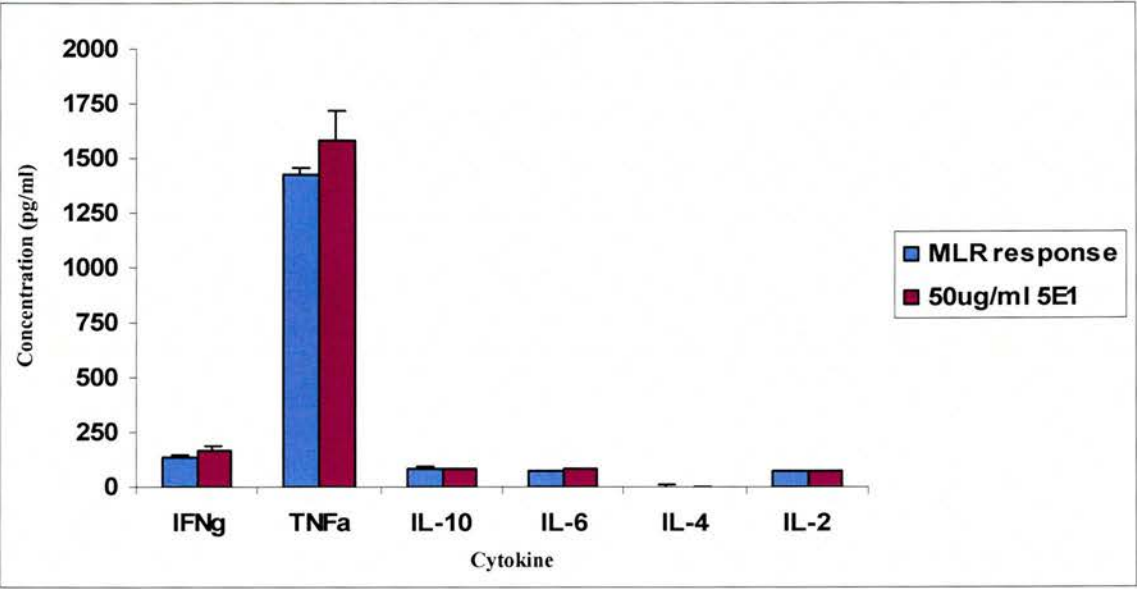


FIGURE 6.9

**MLR proliferation is not modulated by inhibition of Shh signalling**

Depletion-purified CD4<sup>+</sup> T cells were added to cultures of mitomycin C (MMC)-treated macrophages. 10<sup>4</sup> (10<sub>4</sub>), 5x10<sup>4</sup> (5x10<sub>4</sub>), 10<sup>5</sup> (10<sub>5</sub>) and 5x10<sup>5</sup> (5x10<sub>5</sub>) MMC-treated macrophages were used to stimulate the CD4<sup>+</sup> T cells and 50ug/ml 5E1 or 10ng/ml ciclosporin (CsA) were added at the initiation of culture. Ciclosporin abrogated the MLR response. 5E1, however, failed to modulate the MLR proliferative response. Results are representative of at least 4 experiments and means and standard deviations are shown.

**FIGURE 6.10**



**FIGURE 6.10**

**The cytokine effector function of CD4+ T cells in an MLR is not modulated by inhibition of Shh signalling**

Depletion-purified CD4+ T cells were added to cultures of mitomycin C (MMC)-treated macrophages. An MLR using  $5 \times 10^4$  MMC-treated macrophages is illustrated. 5E1 failed to abrogate the production of IFN $\gamma$  (IFN $\gamma$ ), TNF $\alpha$  (TNF $\alpha$ ), IL-10, IL-6, IL-4 and IL-2 in the MLR. Results are representative of at least 4 experiments and means and standard deviations are shown

## **6.5 Shh signalling and the effector function of CD4+ T cells purified using positive CD4+ selection**

The results so far presented in this chapter are somewhat at odds with those previously reported in previous studies<sup>20,21</sup>. Although rShh enhanced the cytokine effector function of suboptimally activated T cells, it failed to modulate the proliferation of suboptimally or optimally activated T cells and, indeed, failed to modulate cytokine effector function of the latter. Both Lowrey et al<sup>21</sup> and Stewart et al<sup>20</sup> reported an effect of rShh on T cell proliferation. In Lowrey's study using murine depletion-purified CD4+ T cells, it was shown that rShh enhanced the proliferation of sub-optimally activated CD4+ T cells. Subsequently, in human studies, Stewart et al<sup>20</sup> demonstrated enhanced proliferation by rShh of optimally, but not suboptimally, activated CD4+ T cells in 50% donors studied, attributing this differential response to possible polymorphisms in the Shh pathway. Indeed, Ptc and Smo mutations can result in the constitutive activation of the Shh pathway<sup>24,25,682</sup> and, thus, it is possible that inactivating mutations may also occur, thereby offering a possible explanation for the differential effects seen.

In the present work, the proliferation of neither suboptimally nor optimally activated T cells was enhanced by the addition of rShh. Furthermore, the addition of the monoclonal antibody to Shh, 5E1, failed to modulate the proliferation of activated CD4+ T cells, a finding in contrast to both previous studies<sup>20,21</sup>. Furthermore, in the extended experiments introducing third party antigen presenting cells into the culture, neither the addition of rShh nor the addition of 5E1 modulated T cell proliferation.

Thus, these findings are interesting. That they are odds with the findings of Lowrey et al<sup>21</sup> might possibly be explained on account of the earlier study employing T cells from an inbred mouse population (C57BL/6J). However, that the findings are not entirely in keeping with those of Stewart et al<sup>20</sup> using the same sources of PBMCs and the same methodologies for cell isolation and purification is of some concern. One striking difference between this work and that of Stewart et al<sup>20</sup> and, indeed, that

of Lowrey et al<sup>21</sup> relates to the reported purities for the depletion columns used. In the present work, the purities of CD4+ T cells were in the order of 82-88%. This is in marked contrast to the purities of human CD4+ T cells reported by Stewart et al<sup>20</sup>. Using the same depletion columns to isolate human CD4+ T cells, purities  $\geq 93\%$  were consistently achieved<sup>20</sup>. This discrepancy in the percentage of contaminating non-CD3+CD4+ cells has the potential to skew the results of these experiments. Indeed, if the contaminating cell fraction contains cells which can respond to a Shh signal and/or stimulate a T cell response, it is conceivable that the presence of such cells could have a profound effect on the proliferative or cytokine effector response of the CD4+ T cells present. It is also possible that any effect of rShh on the CD4+ T cells may be overshadowed by its effects on such contaminating cells present in the system.

Thus, the aim of these experiments was to optimise the purity of the CD4+ T cells. It is well recognised that the purities achieved using depletion systems are inferior to those obtained using positive selection techniques<sup>683,684</sup>. Thus, positive selection techniques were employed to generate a CD4+ cell population of higher purity.

PBMCs were prepared from human Buffy coats as described above and in Chapter 2. Following the adherence step, to remove the majority of the monocytes, the non-adherent cells were removed, washed in PBS and counted.  $20 \times 10^7$  cells were reconstituted in MACs buffer and the mixture incubated for 15 minutes at 4°C with magnetic anti-CD4+ beads (Miltenyi Biotech, Bisley, Surrey, UK) according to the manufacturer's instructions. The mixture was washed twice to remove unbound beads and then applied to the selection column placed in a magnetic field. Beads attached to the CD4+ cells are retained by the column whereas non-CD4+ T cells pass through. Following washing, the column was removed from the magnetic field, thus allowing the CD4+ cells to be eluted from the column. The cells were washed and counted as previously.

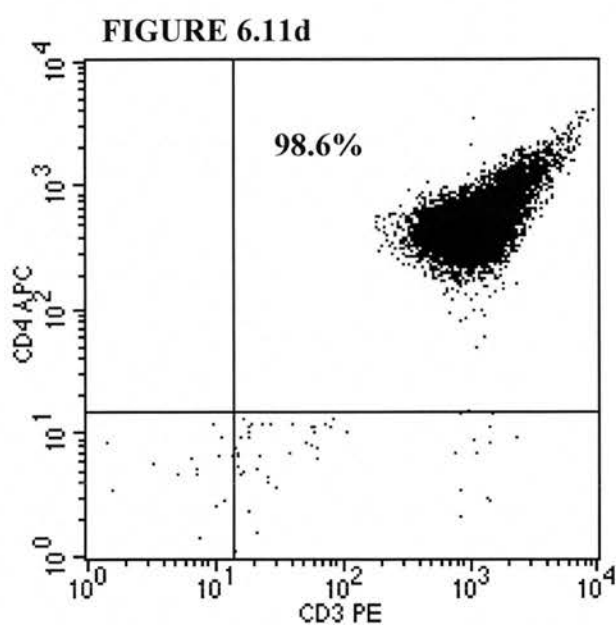
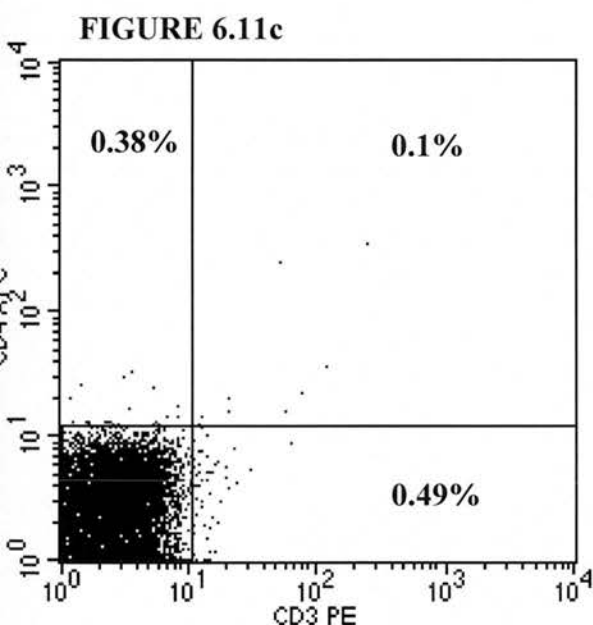
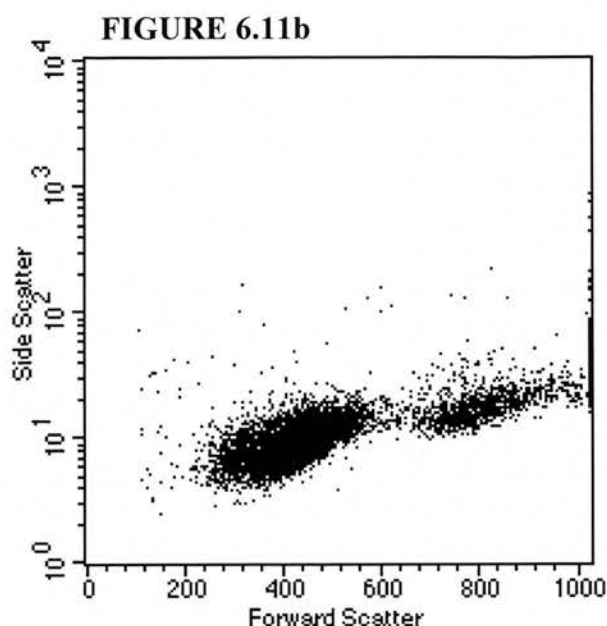
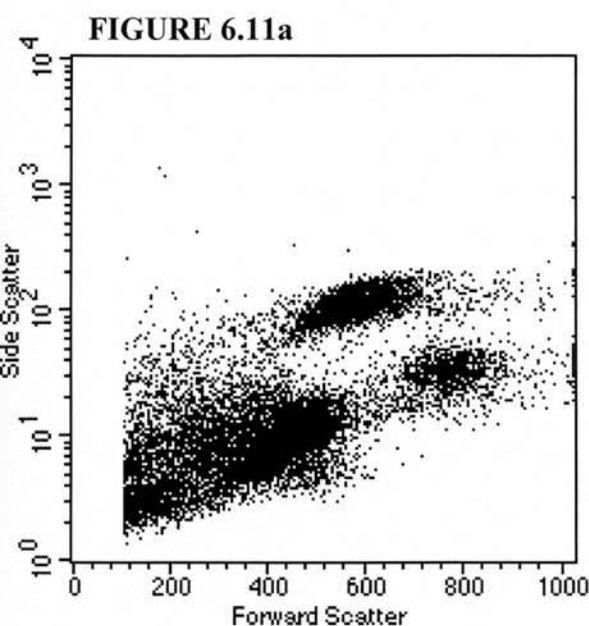


As in the earlier part of this chapter, resting or anti-CD3/anti-CD28 activated CD4+ T cells were incubated either in medium alone or in the presence of 10-1000ng/ml rShh.

The results are shown in Figures 6.11, 6.12 and 6.13. The purities obtained by CD4+ positive selection were better than those obtained using the depletion columns. Indeed, flow cytometry using PE- and APC-labelled mouse anti-human antibodies revealed purities consistently  $\geq 95\%$  (Figure 6.11). These purities were in keeping with those reported by Stewart et al<sup>20</sup> using the R&D depletion columns.

Using positively selected CD4+ T cells, rShh failed to modulate the proliferation of resting T cells, a finding in keeping with previous reports<sup>20,21</sup>. However, despite improved purities, the proliferation of both the suboptimally or optimally activated CD4+ T cells was not modified by the addition of rShh (Figure 6.12).

In cells suboptimally (Figure 6.13), but not optimally (Figure 6.14) activated by anti-CD3/anti-CD28 ligation, IFN- $\gamma$ , TNF $\alpha$ , IL-10, IL-6 and IL-2 were however increased in a dose-dependent fashion by rShh.

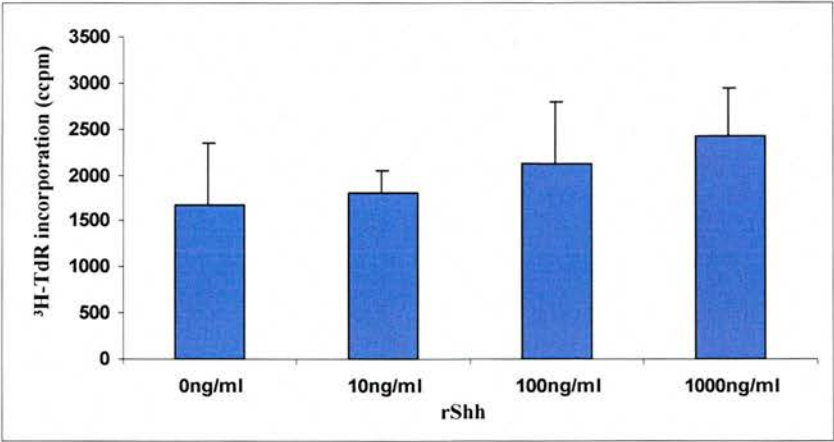


**FIGURE 6.11**

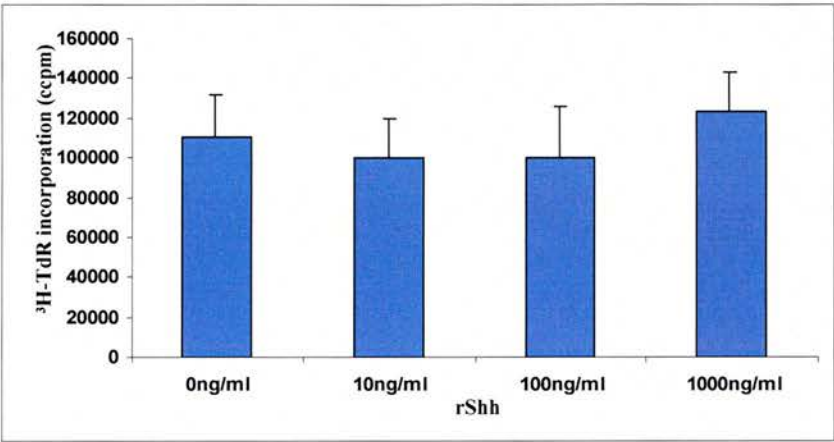
**Representative flow cytometry data demonstrating purities of CD4<sup>+</sup> T cells isolated using positive selection techniques**

Following centrifugation over Lymphoprep, CD4<sup>+</sup> T cells were isolated from PBMCs by magnetic bead separation using CD4 positive selection beads. Representative flow cytometry scatter plots for non-adherent PBMCs (Figure 6.11a), unstained positively selected CD4<sup>+</sup> T cells (Figure 6.11b) and the isotype controls (Figure 6.11c) are shown. Purities of CD3<sup>+</sup>CD4<sup>+</sup> T cells were confirmed using PE-labelled and APC-labelled mouse anti-human antibodies to CD3 and CD4 respectively (Figure 6.11d). Cells purified using positive selection consistently yielded cells with purities  $\geq 95\%$ .

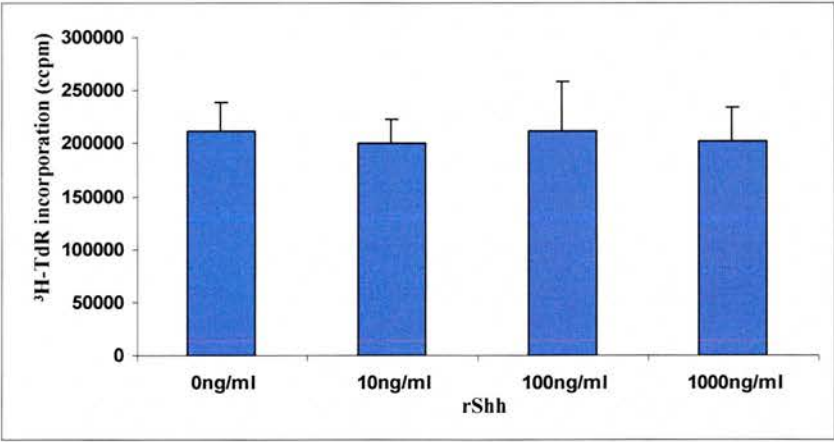
**FIGURE 6.12a**



**FIGURE 6.12b**



**FIGURE 6.12c**



**FIGURE 6.12**  
**rShh fails to modulate the proliferation of resting or activated positively-selected CD4+ T cells**

Resting (Figure 6.12a) or anti-CD3 $\epsilon$ /anti-CD28 activated CD4+ T cells were cultured in the presence or absence of 10-1000ng/ml rShh for 72 hours. Proliferation was assayed by <sup>3</sup>H-TdR incorporation over 18 hours. Irrespective of whether the cells were suboptimally (Figure 6.12b) or optimally (Figure 6.12c) activated, rShh failed to modulate the proliferation of the activated T cells. Results are representative of at least 5 experiments and means and standard deviations are shown.

Figure 6.13a

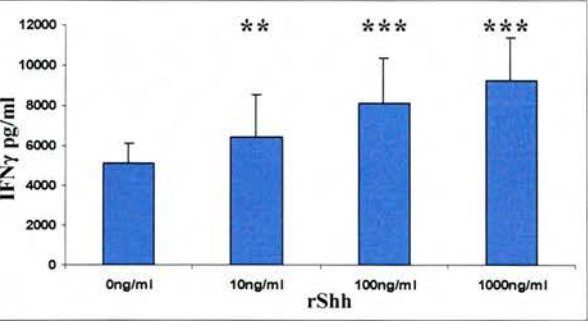


Figure 6.13b

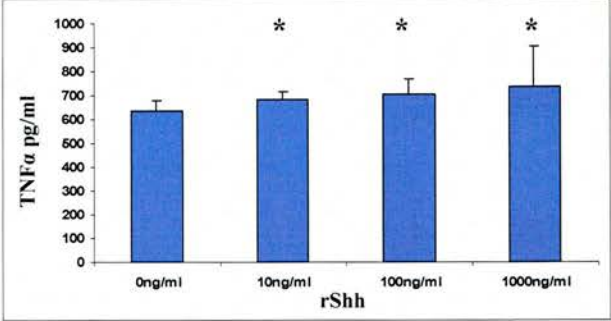


Figure 6.13c

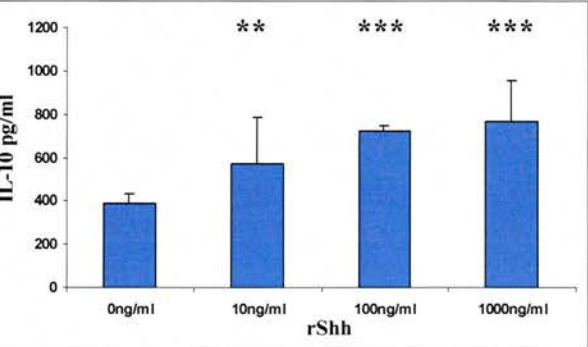


Figure 6.13d

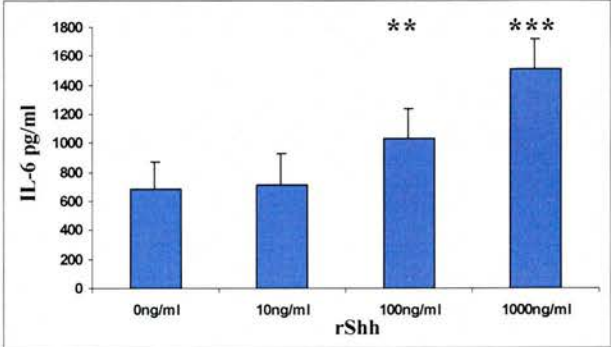


Figure 6.13e

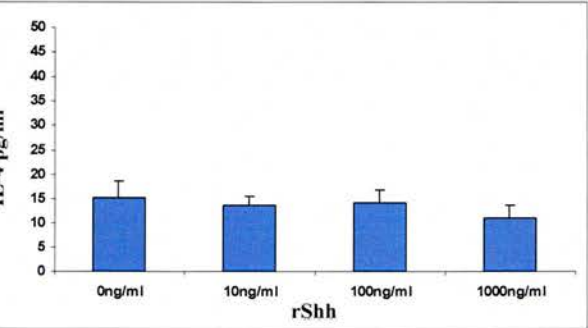


Figure 6.13f

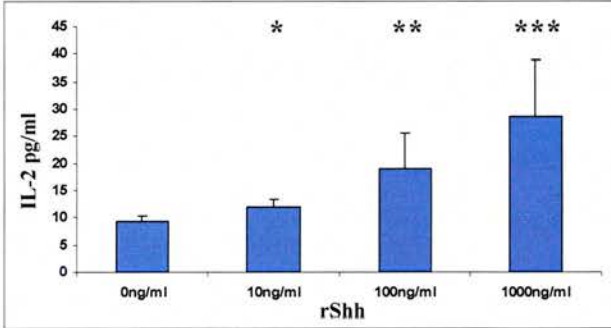


FIGURE 6.13

**rShh upregulates the production of IFN $\gamma$ , TNF $\alpha$ , IL-10, IL-6 and IL-2 from suboptimally activated CD4+ T cells**

Positively-selected CD4+ T cells were suboptimally activated using monoclonal anti-CD3 $\epsilon$  and anti-CD28 antibodies at 0.2 $\mu$ g/ml and 1 $\mu$ g/ml respectively for 72 hours in the presence and absence of 10-1000ng/ml rShh. Supernatants were analysed for cytokine production using cytometric bead arrays. rShh upregulated the production of IFN $\gamma$  (a), TNF $\alpha$  (b), IL-10 (c), IL-6 (d) and IL-2 (f). Conversely, IL-4 was not modulated by rShh (e) For the purposes of statistical analysis, treated CD4 T cells were compared to untreated controls using a two-tailed paired t-test. Results shown are representative of at least 5 donor experiments and means and standard deviations are shown.

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

Figure 6.14a

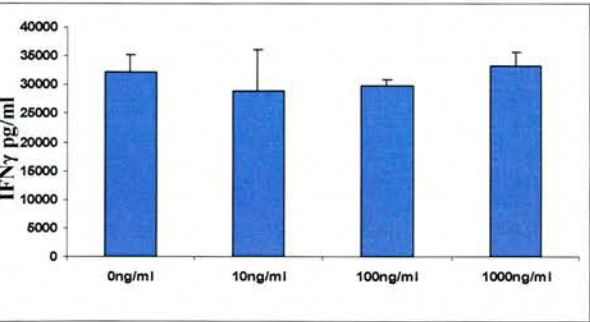


Figure 6.14b

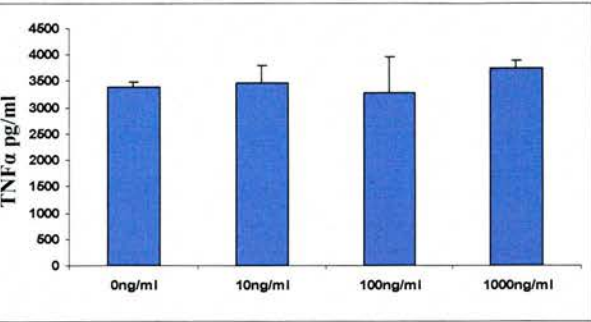


Figure 6.14c

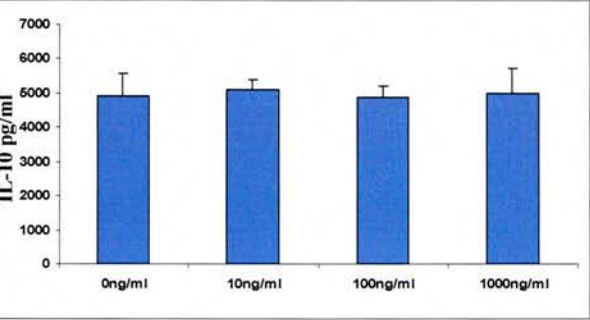


Figure 6.14d

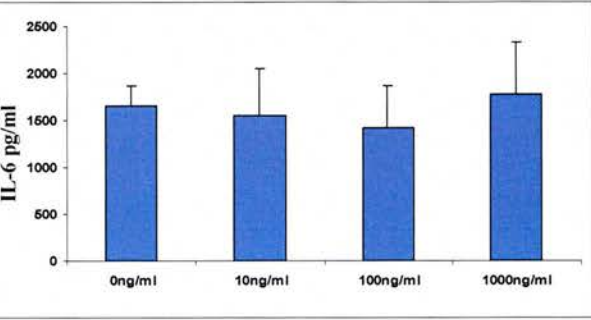


Figure 6.14e

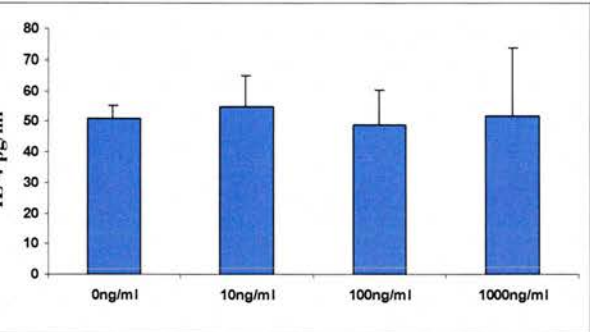
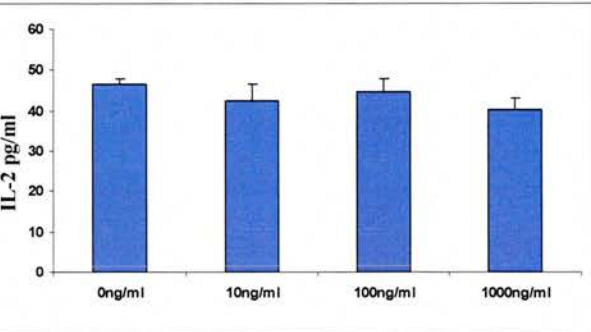


Figure 6.14f



**FIGURE 6.14**  
**rShh fails to modulate the production of IFN $\gamma$ , TNF $\alpha$ , IL-10, IL-6, IL-4 and IL-2 from optimally activated CD4+ T cells**

Positively-selected CD4+ T cells were optimally activated using monoclonal anti-CD3 $\epsilon$  and anti-CD28 antibodies at 1 $\mu$ g/ml and 5 $\mu$ g/ml respectively for 72 hours in the presence and absence of 10-1000ng/ml rShh. Supernatants were analysed for cytokine production using cytometric bead arrays. rShh failed to modulate the production of IFN $\gamma$  (a), TNF $\alpha$  (b), IL-10 (c), IL-6 (d), IL-4 (e) and IL-2 (f). For the purposes of statistical analysis, treated CD4 T cells were compared to untreated controls using a two-tailed paired t-test. Results shown are representative of at least 5 donor experiments and means and standard deviations are shown.



## 6.6 Shh and cell sorted CD3+CD4+ cells

Although the use of the CD4+ positive selection beads produced better purities and, indeed, better yields, of CD4+ T cells, rShh failed to modulate the proliferation of CD4+ T cells. Furthermore, the effect on cytokine effector function mirrored those observed in depletion-purified cells i.e. increased in suboptimally, but not optimally, activated cells. Although this finding supported the earlier findings of this chapter using depletion-purified cells, one point of concern is the observation that the use of CD4+ positive selection appeared to be associated with an element of CD4+ T cell activation, as evidenced by relatively high background levels of proliferation and cytokine production in the unstimulated CD4+ T cells. Indeed, this effect has been described previously in positively selected T cells<sup>685</sup>. Clearly, this has implications, not least because any possible effect relating to the rShh may be masked by this activation.

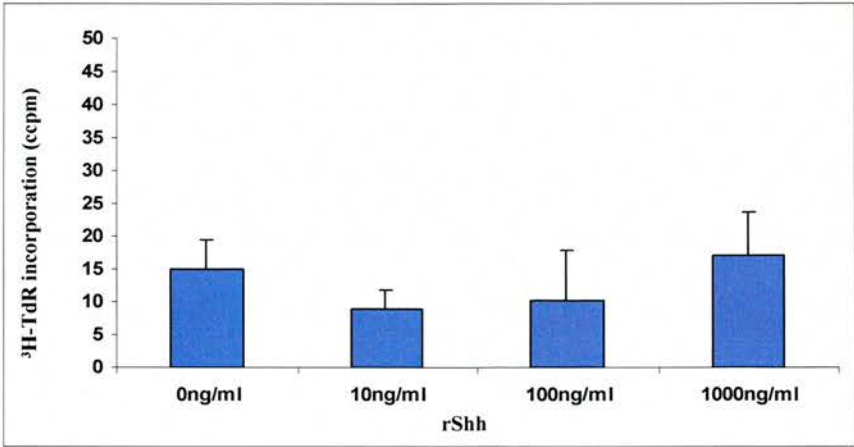
Thus, in a further attempt to generate a highly purified, but not pre-activated, CD4+ T cell population, FACS cell sorting was employed. PBMCs were prepared and the depletion columns (R&D Systems) utilised as previously to isolate a crude population of CD4+ T cells with purities in the range 82-88% as described above in Section 6.2.1. These cells were then incubated in the presence of an excess of PE- and APC- mouse anti-human CD3 and CD4 antibodies respectively. Following washing to remove unbound antibody, the cells were sorted by Shonna McCall (flow cytometry technician, University of Edinburgh) using a FACS Vantage cell sorter (BD Biosciences) to produce a highly purified population of CD3+CD4+ T cells. The purities achieved using this method were consistently  $\geq 98.8\%$ .

Resting or activated CD3+CD4+ T cells were then incubated in the presence or absence of 10-1000ng/ml rShh. As previously, cytokines were removed after 72 hours for evaluation by Th1/Th2 CBA and proliferation was assessed by <sup>3</sup>H-TdR incorporation. The experiment was repeated in 5 donors.

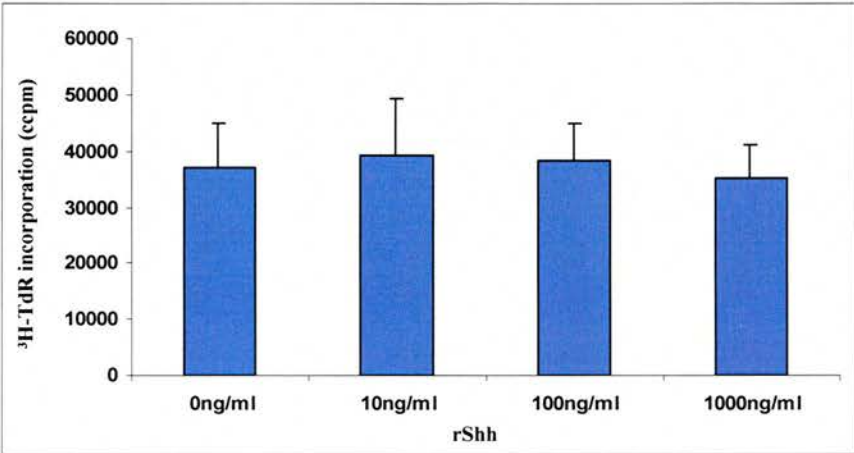


The results are shown in Figures 6.15, 6.16 and 6.17. As previously in this chapter, the proliferation of resting, suboptimally and optimally activated, highly purified, CD4<sup>+</sup> T cells failed to be modulated by the addition of rShh (Figure 6.15). However, cytokine production was modulated, with Shh effecting an upregulation in IFN $\gamma$ , TNF $\alpha$ , IL-10, IL-6 and IL-2 in both suboptimally (Figure 6.16) and optimally (Figure 6.17) activated CD4<sup>+</sup> T cells. IL-4 production was not modulated by the addition of rShh.

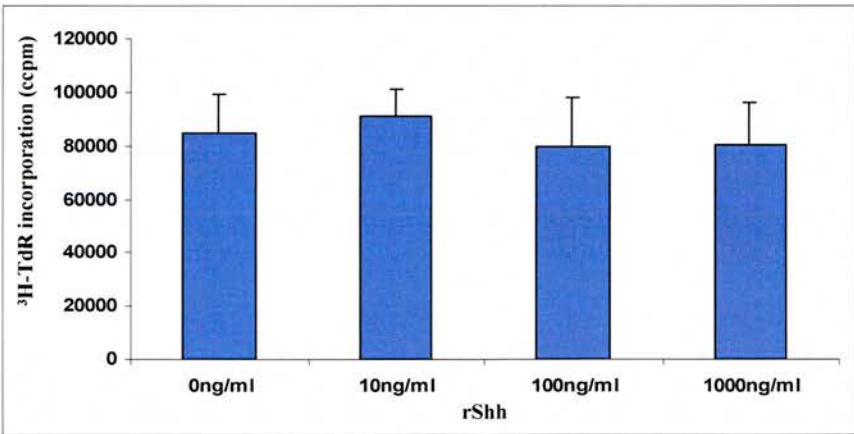
**FIGURE 6.15a**



**FIGURE 6.15b**



**FIGURE 6.15c**



**FIGURE 6.15**  
**rShh fails to modulate the proliferation of resting or activated cell-sorted CD4+ T cells**

Resting (Figure 6.15a) or anti-CD3 $\epsilon$ /anti-CD28 activated CD4+ T cells were cultured in the presence or absence of 10-1000ng/ml rShh for 72 hours. Proliferation was assayed by <sup>3</sup>H-TdR incorporation over 18 hours. Irrespective of whether the cells were suboptimally (Figure 6.15b) or optimally (Figure 6.15c) activated, rShh failed to modulate the proliferation of the activated T cells. Results are representative of at least 4 experiments and means and standard deviations are shown.

Figure 6.16a

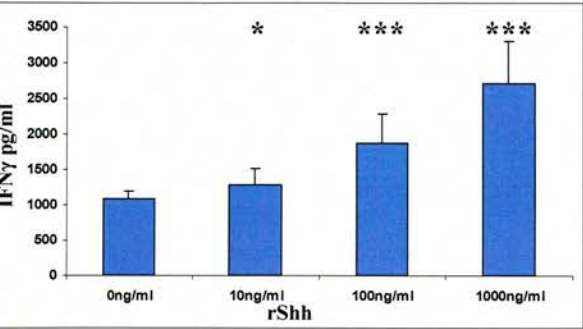


Figure 6.16b

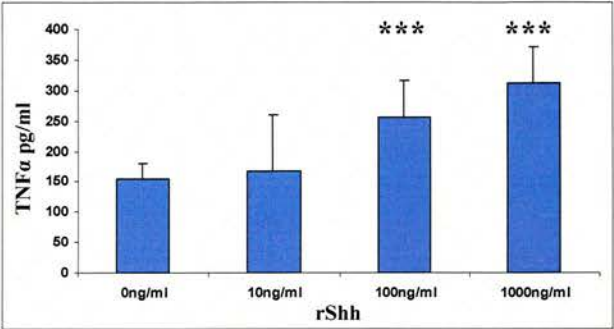


Figure 6.16c

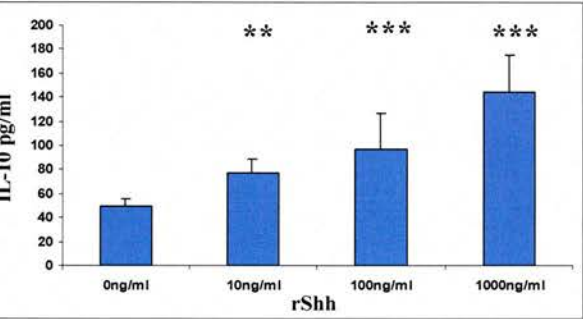


Figure 6.16d

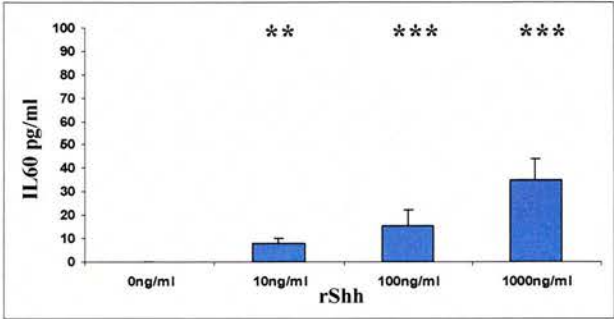


Figure 6.16e

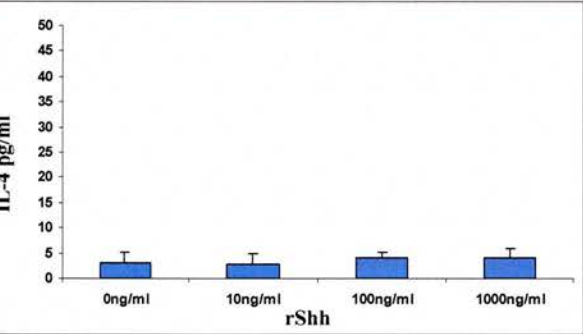


Figure 6.16f

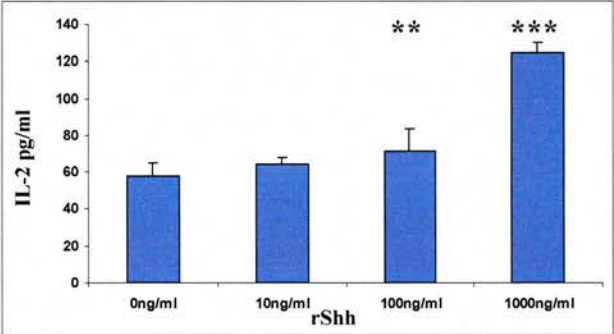


FIGURE 6.16

**rShh upregulates the production of IFN $\gamma$ , TNF $\alpha$ , IL-10, IL-6 and IL-2 from suboptimally activated cell-sorted CD4 $^{+}$  T cells**

Cell-sorted CD4 $^{+}$  T cells were suboptimally activated using monoclonal anti-CD3 $\epsilon$  and anti-CD28 antibodies at 0.2 $\mu$ g/ml and 1 $\mu$ g/ml respectively for 72 hours in the presence and absence of 10-1000ng/ml rShh. Supernatants were analysed for cytokine production using cytometric bead arrays. rShh upregulated the production of IFN $\gamma$  (a), TNF $\alpha$  (b), IL-10 (c), IL-6 (d) and IL-2 (f). Conversely, IL-4 was not modulated by rShh (e) For the purposes of statistical analysis, treated CD4 T cells were compared to untreated controls using a two-tailed paired t-test. Results shown are representative of 5 donor experiments and means and standard deviations are shown.

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

Figure 6.17a

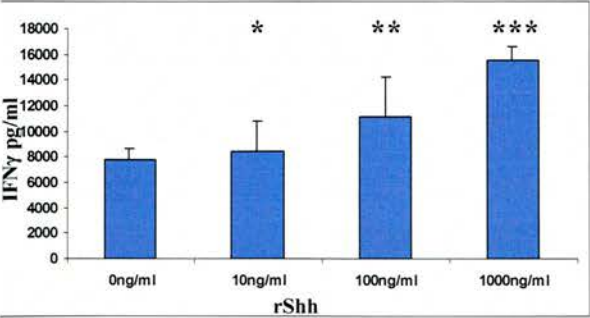


Figure 6.17b

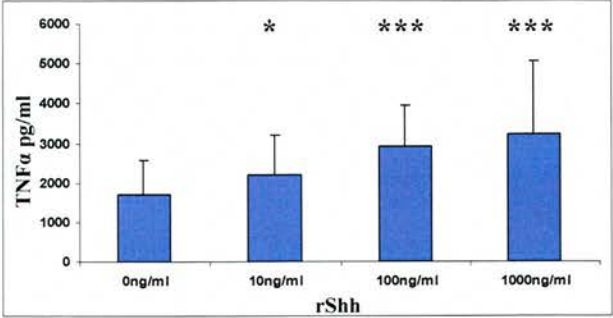


Figure 6.17c

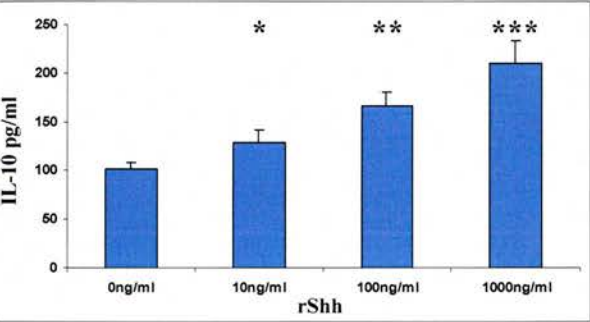


Figure 6.17d

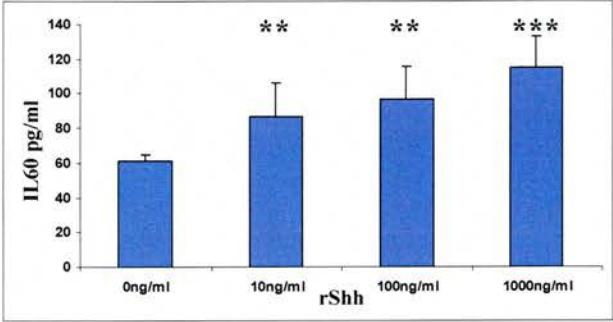


Figure 6.17e

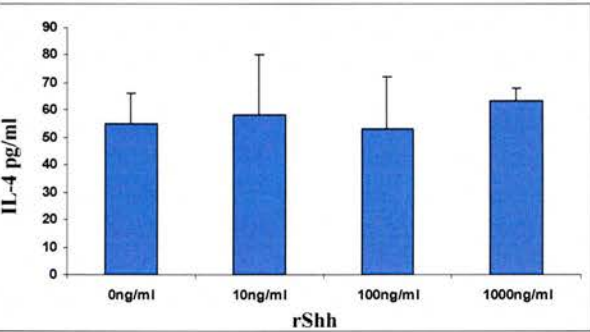


Figure 6.17f

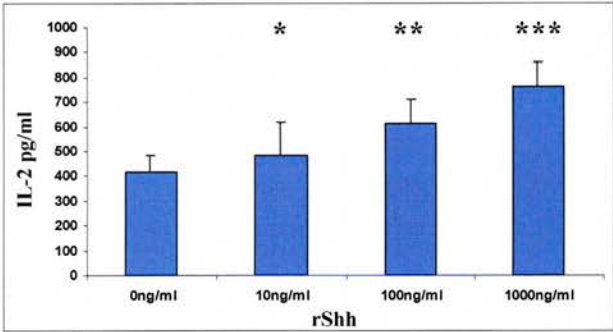


FIGURE 6.17

**rShh upregulates the production of IFN $\gamma$ , TNF $\alpha$ , IL-10, IL-6 and IL-2 from optimally activated cell-sorted CD4 $^{+}$  T cells**

Cell-sorted CD4 $^{+}$  T cells were optimally activated using monoclonal anti-CD3 $\epsilon$  and anti-CD28 antibodies at 1 $\mu$ g/ml and 5 $\mu$ g/ml respectively for 72 hours in the presence and absence of 10-1000ng/ml rShh. Supernatants were analysed for cytokine production using cytometric bead arrays. rShh upregulated the production of IFN $\gamma$  (a), TNF $\alpha$  (b), IL-10 (c), IL-6 (d) and IL-2 (f). Conversely, IL-4 was not modulated by rShh (e) For the purposes of statistical analysis, treated CD4 T cells were compared to untreated controls using a two-tailed paired t-test. Results shown are representative of 5 donor experiments and means and standard deviations are shown.

\*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$

## 6.7 Discussion

The work presented in this chapter has aimed to delineate further the effect of rShh on activated CD4<sup>+</sup> T cell effector function.

### 6.7.1 Shh and T cell effector function

This work followed 2 recent publications from our group. The first, using murine CD4<sup>+</sup> T cells, reported that rShh enhances the proliferation of sub-optimally activated murine CD4<sup>+</sup> T cells<sup>21</sup>, an effect demonstrated to be mediated via the enhanced entry of activated T cells into the cell cycle. Subsequently, Stewart et al<sup>20</sup> demonstrated that rShh enhanced the proliferation of optimally activated, but not sub-optimally activated human PBMC-derived CD4<sup>+</sup> T cells, in 50% donors studied. Furthermore, the work of Stewart et al<sup>20</sup> demonstrated that the addition of rShh differentially modulated the cytokine effector response of optimally activated human CD4<sup>+</sup> T cells. Most notably, IFN- $\gamma$ , IL-2, IL-5 and IL-10 were upregulated in these cells.

Both Lowrey et al<sup>21</sup> and Stewart et al<sup>20</sup> also reported on the effect of the monoclonal antibody, 5E1, on cultures of activated CD4<sup>+</sup> T cells. Lowrey et al<sup>21</sup> demonstrated that 5E1 abrogated the proliferation of suboptimally activated CD4<sup>+</sup> T cells. Stewart et al<sup>20</sup>, using optimally activated human depletion-enriched CD4<sup>+</sup> T cells, demonstrated similar abrogation of proliferation, but in only 50% donors studied. In those donors where an abrogated proliferative response was observed, 5E1 was also shown to inhibit the production of IFN $\gamma$  and IL-2. Conversely, IL-10 and IL-5 production were not abrogated by the addition of 5E1.

Thus, together, the results from these 2 studies would suggest that Shh signalling may be important in modulating both the proliferation and cytokine effector function of adult peripheral CD4<sup>+</sup> T cells, although the reasons for the differential response of human and murine T cells is not clear. In attempting to reproduce these findings in the human system, CD4<sup>+</sup> depletion using negative selection affinity columns (R&D



Systems) was employed as previously<sup>20</sup>. However, in the work presented here, depletion-enriched CD4<sup>+</sup> T cell proliferation failed to be modulated by the addition of rShh in any of the donors studied. An effect of rShh on cytokine effector function was observed but only when suboptimal activation was used. Interestingly, when a third party antigen presenting cell was substituted for anti-CD3/anti-CD28 activation, neither proliferation nor cytokine production were modulated by the addition of rShh. Furthermore, the addition of 5E1 to activated T cells or MLRs failed to modulate either proliferation or cytokine effector function in this system. In light of these results and the disparity from those previously reported<sup>20,21</sup>, it was necessary to re-evaluate why these results were not in keeping with the earlier reports. One possible reason for the differences may have been due to differences in the reported purities. Indeed, in the present work, using depletion techniques alone resulted in a population of cells with 82-88% purity, far lower than those previously described using depletion-enrichment techniques for both murine<sup>21</sup> and human<sup>20</sup> CD4<sup>+</sup> T cells. In both of these studies, purities were reported to be consistently  $\geq 93\%$ . Therefore, it was necessary to further optimise the purity of the CD4<sup>+</sup> T cells used for culture. In the first instance, positive selection techniques were employed. Such techniques generated high purities and yields of CD4<sup>+</sup> T cells although it was readily apparent that positive selection was inherently complicated by the induction of T cell activation as evidenced by high background levels of proliferation and cytokine activity in resting T cells. Subsequently, CD4<sup>+</sup> T cell depletion followed by FACS cell sorting was employed to produce CD4<sup>+</sup> T cells of high purity (consistently  $>98.8\%$ ). In contrast to the results using positively selected CD4<sup>+</sup> T cells, the background activity of the resting cells was in keeping with that observed using depleted T cells alone.

Interestingly, irrespective of the method used to purify the CD4<sup>+</sup> T cells, this work has failed to demonstrate an effect of rShh on the proliferative response of either sub-optimally or optimally activated CD4<sup>+</sup> T cells. This is in marked contrast to the findings in the mouse where rShh maximally augmented CD4<sup>+</sup> T cell proliferation at 100ng/ml rShh<sup>21</sup>. Similarly in the work of Stewart et al<sup>20</sup>, enhanced proliferation was maximal at 100ng/ml, albeit that this effect was demonstrated in only 50% donors



studied. It is not clear why the present work has failed to demonstrate the changes in proliferation previously reported by Lowrey et al<sup>21</sup> and Stewart et al<sup>20</sup>. However, that the effects on proliferation and other markers of T cell activation may be dissociated has been described previously in the literature. Indeed, there are a number of reports suggesting that the use of proliferation as a marker of T cell activity may mask changes in T cell effector function<sup>686-689</sup>. The changes in cytokine effector function seen here in response to rShh in the absence of changes in proliferation would largely be in keeping with these reports. In this regard, a further interesting point arises. In the work of Stewart et al<sup>20</sup>, although the optimal effect on proliferation of CD4+ T cells occurred at 100ng/ml, the effect of rShh on cytokine effector function was maximal at 1000ng/ml, the latter finding consistent with the results presented in this chapter. Why this should be the case remains unclear although this point will be raised again later in this discussion.

In this study, CD4+ T cells have been either suboptimally activated or optimally activated using anti-CD3 $\epsilon$  and anti-CD28 antibodies in keeping with the work of Lowrey et al<sup>21</sup> and Stewart et al<sup>20</sup> respectively. Interestingly, rShh only modulated the cytokine effector function of both suboptimally and optimally activated T cells when highly purified cell sorted cells were used. In contrast, when either depletion-purified or positively selected CD4+ T cells were used, only those cells suboptimally activated by anti-CD3/anti-CD28 responded to rShh through altered cytokine effector function and the cytokine profile induced by optimal anti-CD3/anti-CD28 activation was not augmented by the addition of rShh. Why this is the case is not clear. It may be that rShh fails to augment the effector function of T cells when the cells are already highly activated. Indeed, the levels of T cell activity (in terms of proliferation and background cytokine release) in depletion purified and positively selected T cells were generally higher than when cell sorted cells were used. It may be that the presence of contaminating APCs in the depletion-enriched cells and the inherent activation of T cells together with an albeit small number of contaminating non-CD4+ cells in cells purified by positive selection techniques contributed to this relatively high level of background activation. In keeping with this possibility is the finding that when stimulating third party APCs were substituted for anti-CD3 anti-

CD28 stimulation, rShh failed to augment the cytokine activity of the T cells. Thus, it may be that when the CD4<sup>+</sup> T cells are already highly activated, the effect of a further possible stimulatory source such as rShh, may be masked by the highly activated state of the T cells. Conversely, highly purified CD4<sup>+</sup> T cells, suboptimally or optimally activated by anti-CD3 and anti-CD28 antibodies, but not by the presence of additional contaminating cells may well retain the ability to respond to a low concentration of rShh through further increases in cytokine production. This avenue could be further pursued by extending the range of activating stimuli and modulating the relative contributions of TCR ligation and costimulation accordingly. However, the finding that Stewart et al<sup>20</sup> demonstrated effects on T cell proliferation and cytokine effector function only when optimal stimulation was employed would not lend support to this argument.

In this and the previous series of experiments<sup>20,21</sup>, the effect of the monoclonal antibody, 5E1, has been explored. This antibody has been used previously to inhibit the binding of endogenous Shh protein to the Ptc receptor<sup>19-21,147</sup>. Lowrey et al<sup>21</sup> demonstrated that 5E1 inhibited the proliferation of anti-CD3, anti-CD28 activated CD4<sup>+</sup> T cells. Stewart et al<sup>20</sup> demonstrated the same effect in 50% human donors and, further, demonstrated that 5E1 was able to inhibit the upregulation of IFN $\gamma$  and IL-2, but not IL-5 and IL-10, in optimally activated CD4<sup>+</sup> T cells in those donors responding to 5E1. Interestingly, in the present work, it was not possible to demonstrate an abrogating effect of 5E1 on proliferation or cytokine effector function irrespective of whether suboptimal or optimal activation was used. Similarly, 5E1 was ineffective at inhibiting the MLR response, although low dose ciclosporin could abrogate the MLR response. The reasons for these disparities are not readily apparent. It may be that the antibody was not used at high enough concentrations. 5E1 has been shown previously to inhibit Shh binding to Ptc when used at 20 $\mu$ g/ml<sup>20,21</sup>. In the present series of experiments 10-50 $\mu$ g/ml 5E1 was used but, even at high concentrations, an abrogating effect was not demonstrated. Another possibility is that either of the 2 aliquots of 5E1 antibody may have lost functionality, perhaps explaining why other workers in the laboratory<sup>20,21</sup> demonstrated results using this 5E1 in earlier studies which are at odds with the present findings. In light

of these results, preliminary experiments in 2 donors were performed in which cell sorted activated CD4<sup>+</sup> T cells were incubated with 10 $\mu$ M cyclopamine prior to the addition of exogenous rShh (See Appendix 5). Cyclopamine, like 5E1 also failed to modulate the proliferation or cytokine effector function of the T cells. Taken together, these results would suggest that, at least in my hands, inhibition of Shh signalling fails to modulate the proliferation or cytokine effector function of CD4<sup>+</sup> T cells. However, polymorphisms in the Shh signalling pathway exist and, thus, extrapolating conclusions from these experiments performed in only 2 donors should proceed with caution. Future experiments using a larger donor pool would help to further clarify the effect of cyclopamine on the effector function of CD4<sup>+</sup> T cells. Furthermore, other possible explanations exist. For example, it may be that the effect of Shh on CD4<sup>+</sup> T cell activation occurs independently of the Ptc-Smo-Gli pathway and, indeed, effects attributed to Shh signalling independent of the Ptc-Smo-Gli axis have been proposed to occur elsewhere in the literature<sup>121</sup>. This could explain both the inability of 5E1 and cyclopamine to inhibit the cytokine effector response of the T cells in the work presented here.

### **6.7.2 The Nature of the Stimulus**

The results presented in this chapter raise interesting questions regarding the role for Shh signalling in T cells. The original experiments described in the early part of this chapter suggested that rShh enhanced the cytokine effector function of depletion-purified and positively-selected CD4<sup>+</sup> T cells. Notably, IFN- $\gamma$ , IL-2 and IL-10 were shown to be upregulated in keeping with the earlier findings of Stewart et al<sup>20</sup>. Furthermore, it has also been shown that IL-6 and TNF $\alpha$  are upregulated in response to rShh. This pattern of cytokine response is interesting since cytokines typically associated with Th1 (IFN- $\gamma$ , IL-2 and TNF $\alpha$ ) and Th2 (IL-10) responses are generated together with the non-Th1, non-Th2 cytokine IL-6. Given this pattern of cytokines, one possibility is that both T cells and cells contaminating the preparations of CD4<sup>+</sup> T cells may be contributing to some of the cytokine effector response observed. Indeed, it is known that B cells, CD8<sup>+</sup> T cells and monocyte/macrophages express Ptc and, thus, any or all of these cells, if contaminating the CD4<sup>+</sup> T cell

preparations, may be capable of responding to a rShh signal. Furthermore, from the observations of Chapter 4 and the knowledge that endotoxin is an extremely potent stimulus particularly to cells of the monocytic lineage, it was considered extremely important to generate cells of very high purity to eliminate any possible contribution from a small population of contaminating cells, capable of responding to endotoxin contained within the protein preparation used to explore the effect of Shh *in vitro*. Thus, to address these concerns, cell sorting was employed to generate a highly purified population of CD4+ T cells. Of considerable interest was the finding that, in the absence of contaminating cells, the CD4+ T cells still responded to the rShh through enhanced cytokine production, the profiles seen being similar, albeit of lower magnitude, to those observed when less pure populations of CD4+ T cells were used. Thus, from these results, it would seem that T cells are capable of responding to the rShh used to stimulate them through the production of a variety of different cytokines. In this regard, reports suggesting that the Ptc/Smo receptor complex may be associated with lipid rafts<sup>35,56</sup> are interesting since the importance of lipid rafts in T cell activation is becoming increasingly well understood<sup>386,690-692</sup> and this may provide a mechanism by which Shh signalling may be integral to T cell signalling and effector function. Furthermore, the finding that phosphatidylinositol 3-kinase activity may be triggered by Shh signalling<sup>65</sup> may provide further evidence for a link between Shh signalling and T cell effector processes.

Nevertheless complex issues remain. For example, can we be certain that the response of CD4+ T cells to rShh is in fact a response to Shh itself? Indeed, in the present work it has not been possible to confirm specificity of the response since neither 5E1 nor cyclopamine appeared to modulate the proliferative or cytokine effector response of activated CD4+ T cells, irrespective of whether suboptimal or optimal activation conditions were employed. Furthermore, the findings from Chapter 4 together with several reports demonstrating TLR expression on T cells<sup>693-698</sup> adds a further interesting dimension to this discussion. Most notably, the presence of TLRs on T cells raises the possibility that T cells can respond to LPS and other bacterial components present within the recombinant Shh protein used in these experiments. Although the concentrations of LPS present in the rShh are low, its

presence precludes certainty regarding the role of Shh in these cultures. It is also of importance that whilst LPS is likely to be one of the most potent of the contaminants present, other bacterial contaminants are likely to include bacterial DNA and outer wall proteins such as porins and lipid A-associated proteins, fimbrial proteins, protein A and lipoproteins all of which are capable of inducing cellular activation, via a range of TLRs, particularly including TLR4, TLR2, and TLR9<sup>699</sup>. Such contaminants are not readily detectable using the LAL endotoxin assays and thus are difficult to quantify. This knowledge is important given that, in a number of studies, activation of T cells using bacterial products such as LPS has been demonstrated<sup>700-702</sup>.  $\gamma\delta$  T cells proliferate in response to activation by *E coli* or native lipid A<sup>696,703,704</sup> and regulatory CD4+CD25+ T cells upregulate activation markers and proliferate in response to LPS<sup>697</sup>, the latter effect shown to be augmented by TCR triggering and IL-2 stimulation. In a further study, activation of T cells using TLR3 and TLR9 agonists was shown to result in enhanced activated CD4+ T cell survival without augmenting proliferation<sup>693</sup>.

In light of the findings from this chapter, several other reports are also of interest. For example, resting murine CD3+ T cells express *TLR2* and *TLR4* mRNA<sup>695</sup> and TCR engagement using anti-CD3 $\epsilon$  antibodies and cytokines such as IL-2 have been shown to rapidly induce *TLR2* expression in T cells, an effect involving various LPS- and cytokine-sensitive transcription factors including NF- $\kappa$ B, CREB and STAT<sup>694,695</sup>. Thus, it can be seen how recombinant Shh containing the TLR2 agonist bacterial lipoprotein, which closely associates with LPS, might stimulate T cell events downstream of TLR2 activation. Indeed, recent reports from Liew et al<sup>698</sup> would lend significant support to this notion. Using highly purified anti-CD3 activated CD4+ human T cells, Liew et al<sup>698</sup> demonstrated the expression of mRNA and intracellular proteins of TLR2, TLR4 and MD2. Interestingly, naïve cells failed to express CD14. Significant levels of cell surface expression of TLR2 and TLR4 were shown to peak between 12 and 72 hours following activation demonstrated using a combination of kinetic and immunofluorescence studies. The immunofluorescence studies further helped delineate the pattern of expression of TLRs on T cells. Of note were the findings that, not only were all T cells in a highly purified population positive for the



TLRs, but also TLRs appeared to polarise on the T cells. Whether this reflects the requirement of TLRs for lipid rafts for effective function in a similar manner to the TCRs is not clear. Interestingly, Liew et al<sup>698</sup> demonstrated that, in anti-CD3 activated CD4+ T cells, proliferation was enhanced and cytokine effector function modulated by the addition of the TLR2 agonist, bacterial lipoprotein. Bacterial lipoprotein was shown to markedly enhance the production of IFN $\gamma$ , IL-2 and TNF $\alpha$  by anti-CD3 activated CD4+ T cells. These results prove particularly interesting in light of the results presented in this chapter where the addition of rShh modulated the production of these and other cytokines.

In a further report, both Lipid A and LPS have been shown to upregulate IFN $\gamma$  and downregulate IL-4 production from purified murine CD4+ T cells. This is also an interesting finding in the context of the current results since IL-4 failed to be modulated by rShh in any experiments irrespective of the isolation method employed.

Taken together, the findings from these studies highlight the need for alternatives to the use of *E coli*-derived recombinant Shh for the investigation of Shh signalling, in cells perhaps previously disregarded as responders to endotoxin signals. Indeed, although the levels of LPS present within rShh used in the concentration range 10-1000ng/ml are low, that other endotoxic contaminants will invariably be present (including, of note, TLR2, TLR3 and TLR9 agonists), that T cells upregulate TLR2 mRNA in response to TCR ligation thus enhancing subsequent stimulation by TLR2 agonists and that CD4+ T cell cytokine effector response can be directly modulated by bacterial components, ensures that the investigation of Shh signalling in T cells using recombinant proteins will be difficult. Recent evidence suggesting that CD4+ T cells express several TLRs together with data suggesting that synergy can occur between TLRs<sup>348,349</sup> and that TLR4 signalling can induce TLR2 expression<sup>350</sup> would further add to these concerns. Furthermore, that TLR agonists might synergise with Shh itself is a possibility, although further pursuit along this line of enquiry would prove difficult using bacterially-derived recombinant Shh protein.



Distinguishing between effects due to Shh protein from those due to bacterial contaminants relies on specific pathway inhibitors. In the present work, 5E1 failed to modulate either the proliferation or cytokine effector function of activated T cells, suggesting that the effects observed are not specific to the Shh signalling pathway. It is interesting in this regard that Stewart et al<sup>20</sup>, using the 5E1 antibody in cultures of activated T cells, found inhibition of cytokine effector function in approximately 50% donors. In those responding donors, IFN- $\gamma$  and IL-2, but not IL-10 or IL-5 were inhibited by 5E1. Whether this indicates effects of rShh attributable to both a Shh element and a non-specific stimulatory component of the protein used remains to be determined. It is unfortunate that LPS neutralisers such as polymyxin B (PMB), as employed in Chapter 4, are unlikely to be useful since they have an unpredictable effect on lipoproteins and other bacterial components<sup>681,705</sup> which may be of greater importance given the reports of upregulated TLR2 expression in activated CD4+ T cells<sup>694,695</sup>. More clarity would doubtless be achieved through the employment of non-bacterially-derived and endotoxin-free ligands. Such Shh preparations are not currently commercially available.

### 6.7.3 Summary

The work presented in this chapter has explored the effect of rShh on T cell effector function following recent reports in the literature suggesting that rShh enhanced the proliferation and cytokine effector response of anti-CD3 $\epsilon$ /anti-CD28 activated CD4+ T cells. Three different purification techniques (CD4+ depletion, CD4+ positive selection and CD4+ depletion followed by CD4+ CD3+ FACS cell sorting) were employed to generate CD4+ T cells. Irrespective of the technique used, rShh failed to modulate the proliferation of suboptimally or optimally activated CD4+ T cells and, furthermore, rShh failed to have any effect on the proliferation or cytokine effector function of CD4+ T cells utilised in a mixed leukocyte reaction with third party, mitomycin-C treated, macrophages. Conversely, rShh modulated the cytokine effector function of anti-CD3 $\epsilon$ /anti-CD28 activated CD4+ T cells effecting an upregulation of IFN- $\gamma$ , IL-2, IL-6, TNF $\alpha$  and IL-10, these findings broadly in agreement with those previously published. In keeping with the previous work of

Lowrey et al<sup>21</sup> and Stewart et al<sup>20</sup>, the monoclonal antibody, 5E1 was used to determine whether inhibition of the Shh signalling pathway could result in abrogation of the proliferative or cytokine effector response of activated CD4+ T cells. In contrast to these published reports, 5E1 failed to modify the effector function of CD4+ T cells. Furthermore, in preliminary experiments using cyclopamine, a specific inhibitor of the Shh signalling pathway, no effect of cyclopamine on T cell effector function was observed. Accepting that this failure of T cells to respond to 5E1 and cyclopamine may reflect antibody failure or polymorphisms within the Shh signalling pathway, particularly when the experiments using cyclopamine were performed in only 2 donors, other possibilities must also be considered. In particular, recent reports in the literature demonstrating the expression of TLRs in T cells adds an interesting but complicating dimension to the use of bacterially-derived recombinant Shh protein in cultures of T cells.

# **General Discussion and Conclusions**

The highly conserved Sonic hedgehog signalling pathway plays an important role in the determination of cell fate and proliferation during development. To date, the vast majority of the published reports on Shh signalling have concentrated on developmental processes in the embryo. Much less is known regarding possible roles for Shh signalling in the adult. More recently, however, a few reports have emerged to suggest possible roles for Shh signalling in repair and/or remodelling processes and the maintenance of peripheral immunity in the adult. These reports were central to the development of the work presented in this thesis. In this discussion, the evidence leading up to the hypotheses underlying the work will be outlined briefly. The hypotheses and the findings from the experimental work exploring these hypotheses will then be discussed followed by some conclusions relating to the findings. Areas of possible future investigation are also discussed.

Chronic renal allograft nephropathy is a significant cause of allograft loss in the renal transplant recipient. It is a poorly understood process characterised by interstitial fibrosis, tubular dilatation and atrophy, vasculopathy and an inflammatory cell infiltration. The progressive fibrosis that occurs is considered to be the main cause for the gradual deterioration in renal function and graft failure. That the Shh pathway may be of relevance to the aetiology of CAN is suggested by several lines of evidence. Firstly, although Shh expression has not been reported previously in adult kidney, Shh is expressed in the epithelium of the developing metanephric kidney and Hh signalling is thought to play an early role in renal development. Secondly, Stewart et al<sup>149</sup> recently demonstrated the upregulation of Shh in epithelial cells at sites of fibrotic disease in both human fibrotic lung disease and in murine models of fibrotic lung disease, suggesting that the pathway may play a role in the development of fibrosis. Furthermore, components of the Shh pathway have been demonstrated in immune cells recognised to play a role in the pathogenesis of CAN. Both T cells and macrophages are present in the inflammatory cell infiltrate in CAN with macrophage infiltration and activity correlating directly with graft survival. Shh, Ptc and Gli1 expression have been demonstrated in T cells<sup>20,21</sup>, and Ptc expression has also been reported in macrophages<sup>149</sup>. Furthermore, other factors known to be upregulated in CAN may also be upregulated by Shh. These include the growth

factors, Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) and Vascular Endothelial Growth Factor (VEGF).

Another recently identified area in which Shh signalling may be important in the adult is peripheral immunity. Stewart et al<sup>149</sup> provided evidence to suggest that alveolar macrophages express the receptor for Shh, Ptc, thus raising the possibility that macrophages may be capable of responding to a Shh signal. Two further reports in the literature provided evidence that human and murine CD4<sup>+</sup> T cells expressed components of the Shh signalling pathway and that these cells could respond to a Shh signal<sup>20,21</sup>. In the first of these reports, Lowrey et al demonstrated that the addition of exogenous recombinant Shh protein to cultures of activated murine CD4<sup>+</sup> T cells augmented the proliferative capacity of these cells, an effect mediated through enhanced entry into the S/G2 phase of the cell cycle<sup>21</sup>. Furthermore, inhibition of endogenous Shh using the monoclonal antibody, 5E1, abrogated the proliferative response of activated CD4<sup>+</sup> T cells. Subsequently, Stewart et al<sup>20</sup> published a report regarding human CD4<sup>+</sup> T cells. These findings were broadly in keeping with the earlier work of Lowrey et al<sup>21</sup> and suggested that the addition of recombinant Shh to cultures of human CD4<sup>+</sup> T cells enhanced the proliferation of these cells, but that this effect was observed only in 50% of the donors studied. In those responding donors, the enhanced proliferation was associated with the increased production of cytokines, notably IFN- $\gamma$ , IL-2, IL-5 and IL-10. These reports together highlighted a previously unexplored role for Shh signalling in adult peripheral immunity.

Thus, from the above body of evidence, the two central hypotheses to this thesis were drawn. The first of these was that Shh signalling components are expressed in and relevant to normal and CAN kidney. The second hypothesis was that Shh signalling plays a role in peripheral immunity. In the following discussion, the findings from the work exploring these hypotheses will be addressed.

## **Hypothesis 1 – Shh signalling components are expressed in and relevant to normal and CAN kidney.**

Chapters 3 and 4 of this thesis have explored the hypothesis that Shh signalling is relevant to normal and CAN kidney. In Chapter 3, immunohistochemistry and real-time PCR were used to explore the expression of Shh and Ptc by normal and CAN kidney. Of considerable interest was the finding that Shh expression was demonstrated in the proximal tubular epithelium of normal adult human kidney but that, in contrast to the situation in the fibrotic lung, expression was absent in the kidneys affected by CAN. In contrast, Ptc expression could not be demonstrated in the normal kidney but was present on the infiltrating inflammatory cells of the CAN kidney. These findings prompted speculation as to why the Shh expression seen in normal kidney might be absent from CAN kidney. Clearly, one possibility for these observations is that the decline in number of viable tubules characteristically seen in CAN might preclude the detection of Shh expression in the tissue sections, although it is interesting to note that Shh expression was absent even in tissue sections where grossly normal tubular structure was apparently retained. Thus, it was postulated that the Shh expression seen in the normal kidney had been lost at some stage during the lifespan of the allograft and the subsequent investigation presented in Chapter 4 aimed to investigate factors that may modulate epithelial expression of Shh in the context of transplantation. In this regard, both a human renal adenocarcinoma cell line (ACHN) and primary cultures of murine renal epithelial cells were used to explore possible factors affecting Shh expression in normal renal epithelium. Clearly a large number of factors may have impacted on the Shh expression in normal kidney since the transplanted kidney is subjected to a variety of physiological, pathological and iatrogenic insults through the course of its lifespan. With this in mind, the factors chosen for further investigation included the immunosuppressive agents ciclosporin and dexamethasone, and the effects of epithelial cell damage (in the form of either hydrogen peroxide or ultraviolet irradiation). That epithelial damage may modulate Shh expression and cause release of Shh from sites of damage was suggested both by the work of Stewart et al<sup>149</sup> and also by preliminary experiments by another student in the laboratory, Mr Paul Fitch. Furthermore, that Shh may interact with immune



cells at the epithelium-immune cell interface at sites of damage and/or repair lent support to further investigation along this line of enquiry. Two approaches were used to evaluate the effects of the immunosuppressants and damage on renal epithelium; Shh protein and RNA expression were evaluated in the cells in the presence and absence of either the immunosuppressants or the damaging agent and, furthermore, attempts were made to evaluate and quantify any Shh protein release into the culture medium in response to these agents. These experiments were not without their complications. Most notably, although it was interesting to find that ciclosporin, but not dexamethasone, upregulated *Shh* expression in the ACHN cell line, further investigation of this effect in the primary cultures of murine epithelial cells was hampered by an inability to reproducibly detect *Shh* mRNA expression in the cells. Furthermore, attempts to detect and quantify Shh protein release from cells were also inadequate. Few reagents are available commercially for investigation of the Shh pathway; only a murine Shh ELISA is available but this failed to detect Shh in the supernatants of primary murine renal tubular cultures irrespective of the treatment applied to the cells. Since the upregulation of *Shh* mRNA could only be demonstrated in the human ACHN line but not the primary murine cultures (due to difficulties with primers for both real—time and RT-PCR), the relevance of these findings is not immediately clear although possible explanations for these results are discussed in greater detail in Chapter 4. Indeed, at this stage, one can only speculate as to whether Shh is in fact released from renal epithelial cells since the results presented in Chapter 4 currently fail to support such a mechanism. Clearly, as more reagents become available, it may be more feasible to determine whether Shh is upregulated and/or released from epithelial cells *in vivo*.

## Hypothesis 2 - Shh signalling plays a role in peripheral immunity

Chapters 5 and 6 of this thesis have explored the hypothesis that Shh signalling plays a role in peripheral immunity. Specifically, and relevant to the recent reports in the literature identifying peripheral immune cells as targets of Shh signalling, this work has sought to evaluate Shh signalling in macrophages and CD4<sup>+</sup> T cells.

### *Shh and Macrophages*

The work presented in Chapter 5 explored entirely new territory since the expression of Shh signalling pathway components and the possible role that Shh signalling may play in human peripheral blood-derived macrophages had not previously been explored. The results from Chapter 5 demonstrated that such macrophages express Ptc, Smo, Gli1 and Gli2 suggesting that macrophages may be able to respond to a Shh signal. Furthermore, macrophage *Ptc* expression was upregulated in response to recombinant Shh administration suggesting that active signalling through the Shh signalling pathway was occurring. The subsequent experiments investigated the effect of Shh on human macrophages using the same recombinant Shh peptide (rShh) employed in the earlier *in vitro* work of Lowrey and Stewart. The results from these experiments were initially very interesting demonstrating that Shh upregulated a number of proinflammatory cytokines and chemokines. Of particular interest was the observation that certain proinflammatory cytokines such as TNF $\alpha$ , IL-1 and IL-12, factors were not reliably upregulated by rShh whereas others such as IL-6, IL-8, MCP-1, IP-10, RANTES and MIG were upregulated. The other interesting observation was that use of the specific inhibitor of the Shh signalling pathway cyclopamine inhibited the upregulation of IL-6 and MCP-1 but failed to modulate the upregulation of other cytokines in response to Shh. This was an interesting observation that could not readily be explained. Although, IL-6 and MCP-1 did respond to the cyclopamine, the fact that the upregulation of the other cytokines and chemokines evaluated failed to respond to cyclopamine suggested that either parallel Shh signalling pathways were operating, one Smo-dependent and one Smo-independent, or that Shh was not the only driving stimulus present. Given that the recombinant Shh peptide utilised for these experiments was *E coli* derived, one

significant possibility was that endotoxin contamination of the recombinant protein was responsible for at least some of the effects observed. Subsequent experiments using macrophages grown with and without serum, in the presence of an anti-CD14 antibody and in the presence of polymyxin B were, finally, strongly in favour of LPS exerting the effects observed in the macrophages in response to rShh. Why cyclopamine was able to abrogate the response of IL-6 and MCP-1 is not clear, although the use of cyclopamine reconstituted in LAL reagent water rather than serum-containing medium failed to demonstrate abrogation of the Shh-mediated effect, lending support to the possibility that preincubation of the macrophages with serum (and, therefore, endotoxin) containing cyclopamine may somehow have partially desensitised the macrophages to the subsequent Shh administration and the endotoxin contained therein. Thus, although initially very interesting, the subsequent results presented in Chapter 5 strongly favour endotoxin-mediated effects on macrophages rather than effects due to Shh protein itself. Furthermore, of interest were some preliminary results suggesting that exogenous LPS could also upregulate *Ptc* expression, an important observation given that upregulation of *Ptc*, itself a transcriptional target of Shh signalling, has traditionally been used as a readout of Shh signalling pathway activation.

#### *Shh and CD4+ T cells*

The work presented in Chapter 6 attempted to reproduce the experiments reported previously by Stewart et al<sup>20</sup> in human CD4+ T cells. Both Lowrey et al<sup>21</sup> in the murine system and Stewart et al<sup>20</sup> in human cells had demonstrated an augmented proliferation of activated CD4+ T cells, an effect maximal at 100ng/ml rShh. The initial aim of this chapter had been to reproduce these experiments and then to extend the work further to investigate whether the same effect could be demonstrated when a third party antigen presenting cell was substituted for the anti-CD3 $\epsilon$ /anti-CD28 stimulation. However, despite employing 3 different methods to purify the CD4+ T cells, including the depletion techniques used by both Lowrey et al<sup>21</sup> and Stewart et al<sup>20</sup> in their recent publications, it was not possible to demonstrate an effect of rShh on activated CD4+ T cell proliferation in the experiments presented in this thesis. It was also not possible to demonstrate an effect of the monoclonal antibody, 5E1, on

activated CD4<sup>+</sup> T cell proliferation which was in contrast to these earlier publications. Possible reasons for these discrepancies are discussed in Chapter 5. The main positive finding from Chapter 5 was that, in agreement with the earlier work of Stewart et al<sup>20</sup>, rShh modulated the cytokine effector function of anti-CD3 $\epsilon$ /anti-CD28 activated CD4<sup>+</sup> T cells, although it is of interest that it failed to modulate the magnitude or nature of the cytokine profile of the MLRs. It may be that, in these instances, the CD4<sup>+</sup> T cells were in a state of maximal activation, such that the presence of a further stimulus failed to further amplify the T cell effector function. The earlier work of Stewart et al<sup>20</sup> employed ELISAs to demonstrate the upregulation of IFN- $\gamma$ , IL-2, IL-5 and IL-10. The experiments presented in this thesis add to these findings demonstrating that IL-6 and TNF $\alpha$  are also upregulated by rShh. Stewart et al<sup>20</sup> had also shown that 5E1 was able to prevent the upregulation of IFN- $\gamma$  and IL-2, but not IL-10 or IL-5, in 50% donors studied. In the present work, it has not been possible to demonstrate any effect using 5E1. Whether the antibody had lost activity or whether the donors used in this work were simply similar to the non-responders in Stewart's study is not clear. It is interesting to note that in 2 preliminary studies using the teratogenic Shh signalling pathway inhibitor, cyclopamine, no effect on either CD4<sup>+</sup> T cell proliferation or cytokine effector function was demonstrated. Clearly, however, such experiments would have to be repeated using a much larger donor pool before firm conclusions can be drawn from these observations using cyclopamine.

## **Conclusions and Future Directions**

What conclusions can then be drawn from this work? Perhaps the first conclusion would be that Shh is apparently expressed by normal human renal epithelial cells but not epithelial cells in CAN. This novel observation raises a number of interesting questions, not least: what is the relevance of this expression and what causes loss of expression in CAN? As discussed at length in Chapter 1, Shh may perform a number of roles including the maintenance of cell viability and differentiation or possible involvement in endocytic processes across the tubular cells in association with megalin. Further investigation of such roles would be an interesting avenue of future enquiry. Both Shh <sup>-/-</sup> and Ptc <sup>-/-</sup> mutations in mice are embryonically lethal and

death occurs prior to the development of the kidneys. However, it would be possible to explore the effect of Shh signalling pathway blockade using *in vivo* administration of the 5E1 antibody. Blockade of active signalling followed initially by simple evaluation of renal architecture using immunohistochemistry and electron microscopy would allow analysis of gross changes in structure to be observed. As mentioned previously, further evaluation *in vitro* would be aided greatly by better reagents for evaluating the Shh signalling pathway. As raised in Chapter 4, systems, other than the commercially available murine ELISA, for evaluating activation of the Shh pathway are in use in other laboratories albeit that such systems are not yet available commercially. Three such assays include the use of activation of alkaline phosphatase in C3H10T1/2 cells, activation of Ptc transcription in C3H10T1/2 and activation of a Gli-luciferase reporter in Shh-Light2 cells.

The results from Chapters 5 and 6 raise interesting dilemmas regarding the exploration of Shh signalling in immune cells. It is clearly desirable to investigate the role only of Shh protein in such cells where other immune stimulation, such as that provided by bacterial products, may simply cloud the issue. Indeed, that macrophages may not be the only cells capable of responding to such bacterial products adds to these concerns and means that investigation of the effect of Shh when using bacterially-derived recombinant proteins should proceed with caution. Furthermore, this point highlights the need to address the use of serum in *in vitro* culture systems since, as discussed in Chapter 5, serum also contains significant levels of endotoxic activity. Although the influence of serum components on macrophage effector function has been considered previously, the same consideration has not been extended to work with T cells. Given that T cells may be able to respond to such endotoxic contaminants, the significance of using serum in such cultures should not be underestimated. The soluble CD14 content of serum may also be of similar relevance particularly in the context of T cell cultures, a cell type not characterised by high levels of surface CD14 expression.

The avoidance of the use of recombinant proteins necessitates that other alternatives are available. One possibility might be to use tissue-derived Shh for the *in vitro*

studies. This would be particularly useful since tissue-derived Shh is reported to be more bioactive than the recombinant peptide form. Extracting protein from mammalian tissues is not without its difficulties not least since few anti-Shh antibodies are available and most of these are polyclonal antibodies. One laboratory in the USA (that of PA Beachy) has taken some time to produce the octylated (and more active) form of Shh and it may be that collaboration with this laboratory or the development of such techniques in our laboratory may hold the key to the further investigation of Shh signalling using a non-bacterially derived protein. It might be anticipated that such an approach would at least partly circumvent the difficulties associated with interpreting effects seen in response to rShh. There are, however, other approaches that could be used. For example, eliminating the ability of the cells to respond to Shh or bacterial products might be one approach. It is not clear why 5E1 failed to have the same effect as that previously reported in activated T cells by Lowrey and to a lesser extent by Stewart. 5E1 is believed to bind to Shh at an area overlapping that to which Ptc binds and should thus prevent the binding of the Shh to Ptc. Perhaps simply generating fresher antibody might circumvent the problem although the high levels of endotoxin contamination precludes the use of 5E1 in macrophages and, probably, also future T cell studies. Repeating the cyclopamine experiments in more T cell donors would help to further clarify what effect, if any, cyclopamine has on T cell Shh signalling and may be a more reliable way of assessing Shh signalling in these cells. Other methods of eliminating the ability of cells to respond to a Shh signal might include conditionally knocking out Ptc or other components of the signalling pathway, a technique which would be easier to perform in primary T cell cultures or cell lines rather than primary macrophage cultures. An alternative approach if bacterially-derived recombinant proteins are to be used might be to attempt to eliminate the effect of the bacterial components likely to be present. Detection of such components is really only limited to the detection of LPS using the LAL assay and other components are not reliably detected by this assay. This has important implications particularly when TLR4 is not the only TLR expressed on macrophages and T cells and this further hampers the reliability of using TLR knockout mice for the investigation of Shh signalling events.



Indeed, exclusion of a single TLR, such as TLR4 using the C3H/HeJ or C57BL/10ScCr mice, may be more misleading than helpful since more than one TLR is likely to be involved in the cellular response to recombinant proteins. Furthermore, aside from the bacterial components present in the recombinant protein, Shh signalling itself may even involve the TLRs and, thus, the importance of a non-bacterially derived protein for such experiments is firmly reiterated. That at least LPS might be neutralised by agents such as polymyxin B is highlighted in the discussions of Chapters 5 and 6. However, it is emphasised that the elimination of LPS may not be the only issue. That some contribution may be made by other TLR agonists is highlighted also by the fact that polymyxin B abrogated significantly but could not completely eliminate cytokine production from the macrophages. The extra contribution seems unlikely to be Shh in this instance since the use of cyclopamine and PMB together could not prevent cytokine production although the possibility that the Shh and TLR pathways may interact downstream of the TLRs must preclude firm conclusions from this observation. That other TLR agonists are not reliably inhibited by PMB is of specific importance for the investigation of T cell events not least since reports in the literature suggest that TLR2 may be more important in the T cell with TLR2 expression reported to be linked to T cell activation events and sensitising the T cell to further stimuli. It is clearly important that future investigations of T cell responses, like those of macrophages, avoid the use of bacterially-derived proteins containing TLR agonists such as LPS and bacterial lipoproteins.

Aside from the issues highlighted above, the results from Chapters 5 and 6 do raise some interesting questions. For example, what other roles may Shh play, if any, in macrophages and CD4+ T cells? Does the expression of Shh pathway components serve another purpose? Indeed, it has been proposed that Shh may play a role in commissural axon guidance<sup>706</sup> and other studies have lent support for a possible role for hedgehog family members in the control of migration and motility<sup>707</sup>. Whether Shh might play a role in such processes in immune cells is a line of enquiry yet to be pursued. Clearly, there is still much headway to be made in determining the role of Shh signalling in the adult, a process that would be considerably aided by the

availability of non-bacterially derived Shh and improved reagents for the quantification of Shh expression.

## References

1. Nusslein-Volhard,C. & Wieschaus,E. Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287**, 795-801 (1980).
2. Marigo,V. *et al.* Cloning, expression, and chromosomal location of SHH and IHH: two human homologues of the *Drosophila* segment polarity gene hedgehog. *Genomics* **28**, 44-51 (1995).
3. Hammerschmidt,M., Brook,A. & McMahon,A.P. The world according to hedgehog. *Trends Genet.* **13**, 14-21 (1997).
4. Bitgood,M.J. & McMahon,A.P. Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol.* **172**, 126-138 (1995).
5. McMahon,A.P., Ingham,P.W. & Tabin,C.J. Developmental roles and clinical significance of hedgehog signaling. *Curr. Top. Dev. Biol.* **53**, 1-114 (2003).
6. Lopez-Martinez,A. *et al.* Limb-patterning activity and restricted posterior localization of the amino-terminal product of Sonic hedgehog cleavage. *Curr. Biol.* **5**, 791-796 (1995).
7. Ramalho-Santos,M., Melton,D.A. & McMahon,A.P. Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* **127**, 2763-2772 (2000).
8. Vortkamp,A. *et al.* Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273**, 613-622 (1996).
9. Lanske,B. *et al.* PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* **273**, 663-666 (1996).
10. Roberts,D.J. Molecular mechanisms of development of the gastrointestinal tract. *Dev. Dyn.* **219**, 109-120 (2000).
11. Ingham,P.W. & McMahon,A.P. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087 (2001).
12. Clark,A.M., Garland,K.K. & Russell,L.D. Desert hedgehog (Dhh) gene is required in the mouse testis for formation of adult-type Leydig cells and normal development of peritubular cells and seminiferous tubules. *Biol. Reprod.* **63**, 1825-1838 (2000).

13. Goodrich,L.V., Milenkovic,L., Higgins,K.M. & Scott,M.P. Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* **277**, 1109-1113 (1997).
14. Roessler,E. *et al.* Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. *Nat. Genet.* **14**, 357-360 (1996).
15. Chiang,C. *et al.* Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413 (1996).
16. Litington,Y., Lei,L., Westphal,H. & Chiang,C. Sonic hedgehog is essential to foregut development. *Nat. Genet.* **20**, 58-61 (1998).
17. Incardona,J.P., Gaffield,W., Kapur,R.P. & Roelink,H. The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. *Development* **125**, 3553-3562 (1998).
18. Kelley,R.L. *et al.* Holoprosencephaly in RSH/Smith-Lemli-Opitz syndrome: does abnormal cholesterol metabolism affect the function of Sonic Hedgehog? *Am. J. Med. Genet.* **66**, 478-484 (1996).
19. Bhardwaj,G. *et al.* Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat. Immunol.* **2**, 172-180 (2001).
20. Stewart,G.A. *et al.* Sonic hedgehog signaling modulates activation of and cytokine production by human peripheral CD4+ T cells. *J. Immunol.* **169**, 5451-5457 (2002).
21. Lowrey,J.A. *et al.* Sonic hedgehog promotes cell cycle progression in activated peripheral CD4(+) T lymphocytes. *J. Immunol.* **169**, 1869-1875 (2002).
22. Parisi,M.J. & Lin,H. The role of the hedgehog/patched signaling pathway in epithelial stem cell proliferation: from fly to human. *Cell Res.* **8**, 15-21 (1998).
23. Altaba,A. Gli proteins encode context-dependent positive and negative functions: implications for development and disease. *Development* **126**, 3205-3216 (1999).
24. Xie,J. *et al.* Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature* **391**, 90-92 (1998).
25. Reifemberger,J. *et al.* Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res.* **58**, 1798-1803 (1998).
26. Lench,N.J. *et al.* Characterisation of human patched germ line mutations in naevoid basal cell carcinoma syndrome. *Hum. Genet.* **100**, 497-502 (1997).

27. Hahn,H. *et al.* Mutations of the human homolog of *Drosophila* patched in the nevoid basal cell carcinoma syndrome. *Cell* **85**, 841-851 (1996).
28. Pietsch,T. *et al.* Medulloblastomas of the desmoplastic variant carry mutations of the human homologue of *Drosophila* patched. *Cancer Res.* **57**, 2085-2088 (1997).
29. Bumcrot,D.A., Takada,R. & McMahon,A.P. Proteolytic processing yields two secreted forms of sonic hedgehog. *Mol. Cell Biol.* **15**, 2294-2303 (1995).
30. Marti,E., Bumcrot,D.A., Takada,R. & McMahon,A.P. Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375**, 322-325 (1995).
31. Fan,C.M. *et al.* Long-range sclerotome induction by sonic hedgehog: direct role of the amino-terminal cleavage product and modulation by the cyclic AMP signaling pathway. *Cell* **81**, 457-465 (1995).
32. Porter,J.A. *et al.* Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. *Cell* **86**, 21-34 (1996).
33. Porter,J.A. *et al.* The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. *Nature* **374**, 363-366 (1995).
34. Beachy,P.A. *et al.* Multiple roles of cholesterol in hedgehog protein biogenesis and signaling. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 191-204 (1997).
35. Rietveld,A., Neutz,S., Simons,K. & Eaton,S. Association of sterol- and glycosylphosphatidylinositol-linked proteins with *Drosophila* raft lipid microdomains. *J. Biol. Chem.* **274**, 12049-12054 (1999).
36. Simons,K. & Ikonen,E. Functional rafts in cell membranes. *Nature* **387**, 569-572 (1997).
37. Burke,R. *et al.* Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. *Cell* **99**, 803-815 (1999).
38. The,I., Bellaiche,Y. & Perrimon,N. Hedgehog movement is regulated through tout velu-dependent synthesis of a heparan sulfate proteoglycan. *Mol. Cell* **4**, 633-639 (1999).
39. Pepinsky,R.B. *et al.* Identification of a palmitic acid-modified form of human Sonic hedgehog. *J. Biol. Chem.* **273**, 14037-14045 (1998).
40. Stone,D.M. *et al.* The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* **384**, 129-134 (1996).

41. Johnson,R.L. *et al.* Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science* **272**, 1668-1671 (1996).
42. Nakano,Y. *et al.* A protein with several possible membrane-spanning domains encoded by the Drosophila segment polarity gene patched. *Nature* **341**, 508-513 (1989).
43. Hooper,J.E. & Scott,M.P. The Drosophila patched gene encodes a putative membrane protein required for segmental patterning. *Cell* **59**, 751-765 (1989).
44. Goodrich,L.V., Johnson,R.L., Milenkovic,L., McMahon,J.A. & Scott,M.P. Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev.* **10**, 301-312 (1996).
45. Murone,M., Rosenthal,A. & de Sauvage,F.J. Sonic hedgehog signaling by the patched-smoothened receptor complex. *Curr. Biol.* **9**, 76-84 (1999).
46. Alcedo,J., Ayzenzon,M., Von Ohlen,T., Noll,M. & Hooper,J.E. The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell* **86**, 221-232 (1996).
47. Chen,Y. & Struhl,G. In vivo evidence that Patched and Smoothened constitute distinct binding and transducing components of a Hedgehog receptor complex. *Development* **125**, 4943-4948 (1998).
48. McMahon,A.P. More surprises in the Hedgehog signaling pathway. *Cell* **100**, 185-188 (2000).
49. Ingham,P.W., Taylor,A.M. & Nakano,Y. Role of the Drosophila patched gene in positional signalling. *Nature* **353**, 184-187 (1991).
50. Ingham,P.W. Transducing Hedgehog: the story so far. *EMBO J.* **17**, 3505-3511 (1998).
51. Hahn,H., Wojnowski,L., Miller,G. & Zimmer,A. The patched signaling pathway in tumorigenesis and development: lessons from animal models. *J. Mol. Med.* **77**, 459-468 (1999).
52. Incardona,J.P., Gruenberg,J. & Roelink,H. Sonic hedgehog induces the segregation of patched and smoothened in endosomes. *Curr. Biol.* **12**, 983-995 (2002).
53. Johnson,R.L., Milenkovic,L. & Scott,M.P. In vivo functions of the patched protein: requirement of the C terminus for target gene inactivation but not Hedgehog sequestration. *Mol. Cell* **6**, 467-478 (2000).



54. Incardona, J.P. *et al.* Receptor-mediated endocytosis of soluble and membrane-tethered Sonic hedgehog by Patched-1. *Proc. Natl. Acad. Sci. U. S. A* **97**, 12044-12049 (2000).
55. Mastronardi, F.G., Dimitroulakos, J., Kamel-Reid, S. & Manoukian, A.S. Co-localization of patched and activated sonic hedgehog to lysosomes in neurons. *Neuroreport* **11**, 581-585 (2000).
56. Karpen, H.E. *et al.* The sonic hedgehog receptor patched associates with caveolin-1 in cholesterol-rich microdomains of the plasma membrane. *J. Biol. Chem.* **276**, 19503-19511 (2001).
57. Capdevila, J., Pariente, F., Sampedro, J., Alonso, J.L. & Guerrero, I. Subcellular localization of the segment polarity protein patched suggests an interaction with the wingless reception complex in *Drosophila* embryos. *Development* **120**, 987-998 (1994).
58. Denef, N., Neubuser, D., Perez, L. & Cohen, S.M. Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. *Cell* **102**, 521-531 (2000).
59. Alcedo, J., Zou, Y. & Noll, M. Posttranscriptional regulation of smoothened is part of a self-correcting mechanism in the Hedgehog signaling system. *Mol. Cell* **6**, 457-465 (2000).
60. Martin, V., Carrillo, G., Torroja, C. & Guerrero, I. The sterol-sensing domain of Patched protein seems to control Smoothened activity through Patched vesicular trafficking. *Curr. Biol.* **11**, 601-607 (2001).
61. Strutt, H. *et al.* Mutations in the sterol-sensing domain of Patched suggest a role for vesicular trafficking in Smoothened regulation. *Curr. Biol.* **11**, 608-613 (2001).
62. Chen, J.K., Taipale, J., Young, K.E., Maiti, T. & Beachy, P.A. Small molecule modulation of Smoothened activity. *Proc. Natl. Acad. Sci. U. S. A* **99**, 14071-14076 (2002).
63. Binns, W., James, L.F., Shupe, J.L. & Everett, G. A Congenital cyclopian-type malformation in lambs induced by maternal ingestion of a range plant, *Veratrum californicum*. *Am. J. Vet. Res.* **24**, 1164-1175 (1963).
64. Taipale, J. *et al.* Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature* **406**, 1005-1009 (2000).
65. Kanda, S. *et al.* Sonic hedgehog induces capillary morphogenesis by endothelial cells through phosphoinositide 3-kinase. *J. Biol. Chem.* **278**, 8244-8249 (2003).

66. Berman,D.M. *et al.* Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* **425**, 846-851 (2003).
67. van den Brink,G.R. *et al.* Sonic hedgehog regulates gastric gland morphogenesis in man and mouse. *Gastroenterology* **121**, 317-328 (2001).
68. Thomas,M.K., Rastalsky,N., Lee,J.H. & Habener,J.F. Hedgehog signaling regulation of insulin production by pancreatic beta-cells. *Diabetes* **49**, 2039-2047 (2000).
69. Cooper,M.K., Porter,J.A., Young,K.E. & Beachy,P.A. Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* **280**, 1603-1607 (1998).
70. Chen,J.K., Taipale,J., Cooper,M.K. & Beachy,P.A. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev.* **16**, 2743-2748 (2002).
71. Hepker,J., Wang,Q.T., Motzny,C.K., Holmgren,R. & Orenic,T.V. *Drosophila cubitus interruptus* forms a negative feedback loop with patched and regulates expression of Hedgehog target genes. *Development* **124**, 549-558 (1997).
72. Von Ohlen,T. & Hooper,J.E. Hedgehog signaling regulates transcription through Gli/Ci binding sites in the wingless enhancer. *Mech. Dev.* **68**, 149-156 (1997).
73. Forbes,A.J., Nakano,Y., Taylor,A.M. & Ingham,P.W. Genetic analysis of hedgehog signalling in the *Drosophila* embryo. *Dev. Suppl* 115-124 (1993).
74. Sisson,J.C., Ho,K.S., Suyama,K. & Scott,M.P. Costal2, a novel kinesin-related protein in the Hedgehog signaling pathway. *Cell* **90**, 235-245 (1997).
75. Aza-Blanc,P., Ramirez-Weber,F.A., Laget,M.P., Schwartz,C. & Kornberg,T.B. Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89**, 1043-1053 (1997).
76. Akimaru,H. *et al.* *Drosophila* CBP is a co-activator of cubitus interruptus in hedgehog signalling. *Nature* **386**, 735-738 (1997).
77. Theil,T., Kaesler,S., Grotewold,L., Bose,J. & Ruther,U. Gli genes and limb development. *Cell Tissue Res.* **296**, 75-83 (1999).
78. Altaba,A. Catching a Gli-mpse of Hedgehog. *Cell* **90**, 193-196 (1997).
79. Kinzler,K.W. *et al.* Identification of an amplified, highly expressed gene in a human glioma. *Science* **236**, 70-73 (1987).

80. Ruppert,J.M. *et al.* The GLI-Kruppel family of human genes. *Mol. Cell Biol.* **8**, 3104-3113 (1988).
81. Aza-Blanc,P., Lin,H.Y., Altaba,A. & Kornberg,T.B. Expression of the vertebrate Gli proteins in *Drosophila* reveals a distribution of activator and repressor activities. *Development* **127**, 4293-4301 (2000).
82. Hynes,M. *et al.* Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli-1. *Neuron* **19**, 15-26 (1997).
83. Ruppert,J.M., Vogelstein,B. & Kinzler,K.W. The zinc finger protein GLI transforms primary cells in cooperation with adenovirus E1A. *Mol. Cell Biol.* **11**, 1724-1728 (1991).
84. Dahmane,N., Lee,J., Robins,P., Heller,P. & Altaba,A. Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. *Nature* **389**, 876-881 (1997).
85. Roberts,W.M., Douglass,E.C., Peiper,S.C., Houghton,P.J. & Look,A.T. Amplification of the gli gene in childhood sarcomas. *Cancer Res.* **49**, 5407-5413 (1989).
86. Stein,U. *et al.* GLI gene expression in bone and soft tissue sarcomas of adult patients correlates with tumor grade. *Cancer Res.* **59**, 1890-1895 (1999).
87. Park,H.L. *et al.* Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. *Development* **127**, 1593-1605 (2000).
88. Sasaki,H., Nishizaki,Y., Hui,C., Nakafuku,M. & Kondoh,H. Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* **126**, 3915-3924 (1999).
89. Ding,Q. *et al.* Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. *Development* **125**, 2533-2543 (1998).
90. Hardcastle,Z., Mo,R., Hui,C.C. & Sharpe,P.T. The Shh signalling pathway in tooth development: defects in Gli2 and Gli3 mutants. *Development* **125**, 2803-2811 (1998).
91. Motoyama,J. *et al.* Essential function of Gli2 and Gli3 in the formation of lung, trachea and oesophagus. *Nat. Genet.* **20**, 54-57 (1998).
92. Altaba,A. Combinatorial Gli gene function in floor plate and neuronal inductions by Sonic hedgehog. *Development* **125**, 2203-2212 (1998).

93. Vortkamp,A., Gessler,M. & Grzeschik,K.H. GLI3 zinc-finger gene interrupted by translocations in Greig syndrome families. *Nature* **352**, 539-540 (1991).
94. Villavicencio,E.H., Walterhouse,D.O. & Iannaccone,P.M. The sonic hedgehog-patched-gli pathway in human development and disease. *Am. J. Hum. Genet.* **67**, 1047-1054 (2000).
95. Wang,B., Fallon,J.F. & Beachy,P.A. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* **100**, 423-434 (2000).
96. Litington,Y. & Chiang,C. Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. *Nat. Neurosci.* **3**, 979-985 (2000).
97. Alexandre,C., Jacinto,A. & Ingham,P.W. Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins. *Genes Dev.* **10**, 2003-2013 (1996).
98. Hidalgo,A. & Ingham,P. Cell patterning in the Drosophila segment: spatial regulation of the segment polarity gene patched. *Development* **110**, 291-301 (1990).
99. Marigo,V. & Tabin,C.J. Regulation of patched by sonic hedgehog in the developing neural tube. *Proc. Natl. Acad. Sci. U. S. A* **93**, 9346-9351 (1996).
100. Marigo,V., Davey,R.A., Zuo,Y., Cunningham,J.M. & Tabin,C.J. Biochemical evidence that patched is the Hedgehog receptor. *Nature* **384**, 176-179 (1996).
101. Chen,Y. & Struhl,G. Dual roles for patched in sequestering and transducing Hedgehog. *Cell* **87**, 553-563 (1996).
102. Pola,R. *et al.* The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat. Med.* **7**, 706-711 (2001).
103. Pola,R. *et al.* Postnatal recapitulation of embryonic hedgehog pathway in response to skeletal muscle ischemia. *Circulation* **108**, 479-485 (2003).
104. Haraguchi,R. *et al.* Unique functions of Sonic hedgehog signaling during external genitalia development. *Development* **128**, 4241-4250 (2001).
105. Oliver,T.G. *et al.* Transcriptional profiling of the Sonic hedgehog response: a critical role for N-myc in proliferation of neuronal precursors. *Proc. Natl. Acad. Sci. U. S. A* **100**, 7331-7336 (2003).

106. Yoon,J.W. *et al.* Gene expression profiling leads to identification of GLI1-binding elements in target genes and a role for multiple downstream pathways in GLI1-induced cell transformation. *J. Biol. Chem.* **277**, 5548-5555 (2002).
107. Massague,J. TGF-beta signal transduction. *Annu. Rev. Biochem.* **67**, 753-791 (1998).
108. Hogan,B.L. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10**, 1580-1594 (1996).
109. Roberts,A.B. *et al.* Transforming growth factor-beta: multifunctional regulator of differentiation and development. *Philos. Trans. R. Soc. Lond B Biol. Sci.* **327**, 145-154 (1990).
110. Heberlein,U., Wolff,T. & Rubin,G.M. The TGF beta homolog dpp and the segment polarity gene hedgehog are required for propagation of a morphogenetic wave in the Drosophila retina. *Cell* **75**, 913-926 (1993).
111. Laufer,E., Nelson,C.E., Johnson,R.L., Morgan,B.A. & Tabin,C. Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79**, 993-1003 (1994).
112. Ingham,P.W. & Fietz,M.J. Quantitative effects of hedgehog and decapentaplegic activity on the patterning of the Drosophila wing. *Curr. Biol.* **5**, 432-440 (1995).
113. Alvarez,J. *et al.* TGFbeta2 mediates the effects of hedgehog on hypertrophic differentiation and PTHrP expression. *Development* **129**, 1913-1924 (2002).
114. Sanchez-Herrero,E., Couso,J.P., Capdevila,J. & Guerrero,I. The fu gene discriminates between pathways to control dpp expression in Drosophila imaginal discs. *Mech. Dev.* **55**, 159-170 (1996).
115. Basler,K. & Struhl,G. Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. *Nature* **368**, 208-214 (1994).
116. Tabata,T. & Kornberg,T.B. Hedgehog is a signaling protein with a key role in patterning Drosophila imaginal discs. *Cell* **76**, 89-102 (1994).
117. Nishita,M. *et al.* Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer. *Nature* **403**, 781-785 (2000).
118. Wozney,J.M. The bone morphogenetic protein family: multifunctional cellular regulators in the embryo and adult. *Eur. J. Oral Sci.* **106 Suppl 1**, 160-166 (1998).

119. Hogan,B.L. Bone morphogenetic proteins in development. *Curr. Opin. Genet. Dev.* **6**, 432-438 (1996).
120. Bruneau,S. & Rosa,F.M. Dynamo, a new zebrafish DVR member of the TGF-beta superfamily is expressed in the posterior neural tube and is up-regulated by Sonic hedgehog. *Mech. Dev.* **61**, 199-212 (1997).
121. Testaz,S. *et al.* Sonic hedgehog restricts adhesion and migration of neural crest cells independently of the Patched- Smoothened-Gli signaling pathway. *Proc. Natl. Acad. Sci. U. S. A* **98**, 12521-12526 (2001).
122. Ramirez-Weber,F.A., Casso,D.J., Aza-Blanc,P., Tabata,T. & Kornberg,T.B. Hedgehog signal transduction in the posterior compartment of the *Drosophila* wing imaginal disc. *Mol. Cell* **6**, 479-485 (2000).
123. Bokor,P. & DiNardo,S. The roles of hedgehog, wingless and lines in patterning the dorsal epidermis in *Drosophila*. *Development* **122**, 1083-1092 (1996).
124. Chuang,P.T. & McMahon,A.P. Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. *Nature* **397**, 617-621 (1999).
125. Pons,S. & Marti,E. Sonic hedgehog synergizes with the extracellular matrix protein vitronectin to induce spinal motor neuron differentiation. *Development* **127**, 333-342 (2000).
126. McCarthy,R.A., Barth,J.L., Chintalapudi,M.R., Knaak,C. & Argraves,W.S. Megalin functions as an endocytic sonic hedgehog receptor. *J. Biol. Chem.* **277**, 25660-25667 (2002).
127. McCarthy,R.A. & Argraves,W.S. Megalin and the neurodevelopmental biology of sonic hedgehog and retinol. *J. Cell Sci.* **116**, 955-960 (2003).
128. Carpenter,D. *et al.* Characterization of two patched receptors for the vertebrate hedgehog protein family. *Proc. Natl. Acad. Sci. U. S. A* **95**, 13630-13634 (1998).
129. Motoyama,J., Takabatake,T., Takeshima,K. & Hui,C. Ptch2, a second mouse Patched gene is co-expressed with Sonic hedgehog. *Nat. Genet.* **18**, 104-106 (1998).
130. Zaphiropoulos,P.G., Unden,A.B., Rahnama,F., Hollingsworth,R.E. & Toftgard,R. PTCH2, a novel human patched gene, undergoing alternative splicing and up-regulated in basal cell carcinomas. *Cancer Res.* **59**, 787-792 (1999).
131. St Jacques,B. *et al.* Sonic hedgehog signaling is essential for hair development. *Curr. Biol.* **8**, 1058-1068 (1998).



132. Chuang,P.T., Kawcak,T. & McMahon,A.P. Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung. *Genes Dev.* **17**, 342-347 (2003).
133. Raychowdhury,R., Niles,J.L., McCluskey,R.T. & Smith,J.A. Autoimmune target in Heymann nephritis is a glycoprotein with homology to the LDL receptor. *Science* **244**, 1163-1165 (1989).
134. Verroust,P.J. & Christensen,E.I. Megalin and cubilin--the story of two multipurpose receptors unfolds. *Nephrol. Dial. Transplant.* **17**, 1867-1871 (2002).
135. Lundgren,S. *et al.* Tissue distribution of human gp330/megalin, a putative Ca(2+)-sensing protein. *J. Histochem. Cytochem.* **45**, 383-392 (1997).
136. Willnow,T.E., Armstrong,S.A., Hammer,R.E. & Herz,J. Functional expression of low density lipoprotein receptor-related protein is controlled by receptor-associated protein in vivo. *Proc. Natl. Acad. Sci. U. S. A* **92**, 4537-4541 (1995).
137. Kim,P.C., Mo,R. & Hui,C.C. Murine models of VACTERL syndrome: Role of sonic hedgehog signaling pathway. *J. Pediatr. Surg.* **36**, 381-384 (2001).
138. Yu,J., Carroll,T.J. & McMahon,A.P. Sonic hedgehog regulates proliferation and differentiation of mesenchymal cells in the mouse metanephric kidney. *Development* **129**, 5301-5312 (2002).
139. Karavanova,I.D., Dove,L.F., Resau,J.H. & Perantoni,A.O. Conditioned medium from a rat ureteric bud cell line in combination with bFGF induces complete differentiation of isolated metanephric mesenchyme. *Development* **122**, 4159-4167 (1996).
140. Dudley,A.T. & Robertson,E.J. Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos. *Dev. Dyn.* **208**, 349-362 (1997).
141. Zhang,H. & Bradley,A. Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development* **122**, 2977-2986 (1996).
142. Piscione,T.D. *et al.* BMP-2 and OP-1 exert direct and opposite effects on renal branching morphogenesis. *Am. J. Physiol* **273**, F961-F975 (1997).
143. Miyazaki,Y., Oshima,K., Fogo,A., Hogan,B.L. & Ichikawa,I. Bone morphogenetic protein 4 regulates the budding site and elongation of the mouse ureter. *J. Clin. Invest* **105**, 863-873 (2000).

144. Raatikainen-Ahokas,A., Hytonen,M., Tenhunen,A., Sainio,K. & Sariola,H. BMP-4 affects the differentiation of metanephric mesenchyme and reveals an early anterior-posterior axis of the embryonic kidney. *Dev. Dyn.* **217**, 146-158 (2000).
145. Winnier,G., Blessing,M., Labosky,P.A. & Hogan,B.L. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev.* **9**, 2105-2116 (1995).
146. Valentini,R.P. *et al.* Post-translational processing and renal expression of mouse Indian hedgehog. *J. Biol. Chem.* **272**, 8466-8473 (1997).
147. Outram,S.V., Varas,A., Pepicelli,C.V. & Crompton,T. Hedgehog signaling regulates differentiation from double-negative to double-positive thymocyte. *Immunity.* **13**, 187-197 (2000).
148. Sacedon,R. *et al.* Expression of hedgehog proteins in the human thymus. *J. Histochem. Cytochem.* **51**, 1557-1566 (2003).
149. Stewart,G.A. *et al.* Expression of the developmental Sonic hedgehog (Shh) signalling pathway is up-regulated in chronic lung fibrosis and the Shh receptor patched 1 is present in circulating T lymphocytes. *J. Pathol.* **199**, 488-495 (2003).
150. Wang,L.C. *et al.* Regular articles: conditional disruption of hedgehog signaling pathway defines its critical role in hair development and regeneration. *J. Invest Dermatol.* **114**, 901-908 (2000).
151. Warburton,D. *et al.* Do lung remodeling, repair, and regeneration recapitulate respiratory ontogeny? *Am. J. Respir. Crit Care Med.* **164**, S59-S62 (2001).
152. Ferguson,C.M., Miclau,T., Hu,D., Alpern,E. & Helms,J.A. Common molecular pathways in skeletal morphogenesis and repair. *Ann. N. Y. Acad. Sci.* **857**, 33-42 (1998).
153. Vortkamp,A. *et al.* Recapitulation of signals regulating embryonic bone formation during postnatal growth and in fracture repair. *Mech. Dev.* **71**, 65-76 (1998).
154. Murakami,S. & Noda,M. Expression of Indian hedgehog during fracture healing in adult rat femora. *Calcif. Tissue Int.* **66**, 272-276 (2000).
155. Wu,Q., Zhang,Y. & Chen,Q. Indian hedgehog is an essential component of mechanotransduction complex to stimulate chondrocyte proliferation. *J. Biol. Chem.* **276**, 35290-35296 (2001).
156. Jemtland,R., Divieti,P., Lee,K. & Segre,G.V. Hedgehog promotes primary osteoblast differentiation and increases PTHrP mRNA expression and iPTHrP secretion. *Bone* **32**, 611-620 (2003).

157. Kinto,N. *et al.* Fibroblasts expressing Sonic hedgehog induce osteoblast differentiation and ectopic bone formation. *FEBS Lett.* **404**, 319-323 (1997).
158. Spinella-Jaegle,S. *et al.* Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation. *J. Cell Sci.* **114**, 2085-2094 (2001).
159. Ishizuya-Oka,A. *et al.* Thyroid hormone-induced expression of sonic hedgehog correlates with adult epithelial development during remodeling of the *Xenopus* stomach and intestine. *Differentiation* **69**, 27-37 (2001).
160. Dimmler,A. *et al.* Transcription of sonic hedgehog, a potential factor for gastric morphogenesis and gastric mucosa maintenance, is up-regulated in acidic conditions. *Lab Invest* **83**, 1829-1837 (2003).
161. Watkins,D.N. *et al.* Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature* **422**, 313-317 (2003).
162. Reynolds,S.D. *et al.* Conditional clara cell ablation reveals a self-renewing progenitor function of pulmonary neuroendocrine cells. *Am. J. Physiol Lung Cell Mol. Physiol* **278**, L1256-L1263 (2000).
163. Lawson,N.D., Vogel,A.M. & Weinstein,B.M. sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev. Cell* **3**, 127-136 (2002).
164. Krishnan,V. *et al.* Mediation of Sonic hedgehog-induced expression of COUP-TFII by a protein phosphatase. *Science* **278**, 1947-1950 (1997).
165. Pereira,F.A., Qiu,Y., Zhou,G., Tsai,M.J. & Tsai,S.Y. The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. *Genes Dev.* **13**, 1037-1049 (1999).
166. Azuma,H., Heemann,U., Tullius,S.G. & Tilney,N.L. Host leukocytes and their products in chronic kidney allograft rejection in rats. *Transpl. Int.* **7 Suppl 1**, S325-S327 (1994).
167. Cecka,J.M. The UNOS Scientific Renal Transplant Registry. *Clin. Transpl.* 1-14 (1996).
168. Paul,L.C. Surrogate biomarkers of long-term outcome after kidney transplantation. *Ann. Transplant.* **5**, 5-6 (2000).
169. Tilney,N.L., Whitley,W.D., Diamond,J.R., Kupiec-Weglinski,J.W. & Adams,D.H. Chronic rejection--an undefined conundrum. *Transplantation* **52**, 389-398 (1991).

170. Krieger,N.R. *et al.* Chronic allograft nephropathy uniformly affects recipients of cadaveric, nonidentical living-related, and living-unrelated grafts. *Transplantation* **75**, 1677-1682 (2003).
171. Matas,A.J. Is MHC matching as a primary criterion in kidney allocation justified? *Nat. Genet.* **5**, 210-213 (1993).
172. Paul,L.C. Current knowledge of the pathogenesis of chronic allograft dysfunction. *Transplant. Proc.* **31**, 1793-1795 (1999).
173. Hayry,P. *et al.* Chronic allograft rejection. *Immunol. Rev.* **134**, 33-81 (1993).
174. Paul,L.C. Chronic rejection of organ allografts: magnitude of the problem. *Transplant. Proc.* **25**, 2024-2025 (1993).
175. Toussaint,C., Kinnaert,P. & Vereerstraeten,P. Late mortality and morbidity five to eighteen years after kidney transplantation. *Transplantation* **45**, 554-558 (1988).
176. Modena,F.M. *et al.* Progression of kidney disease in chronic renal transplant rejection. *Transplantation* **52**, 239-244 (1991).
177. Viklicky,O. *et al.* TGF-beta1 expression and chronic allograft nephropathy in protocol kidney graft biopsy. *Physiol Res.* **52**, 353-360 (2003).
178. Feehally,J., Harris,K.P., Bennett,S.E. & Walls,J. Is chronic renal transplant rejection a non-immunological phenomenon? *Lancet* **2**, 486-488 (1986).
179. Fellstrom,B., Backman,U., Larsson,E. & Zezina,L. Immunologic and nonimmunologic risk factors of chronic rejection. *Transplant. Proc.* **31**, 1304-1305 (1999).
180. Hancock,W.H. *et al.* Cytokines, adhesion molecules, and the pathogenesis of chronic rejection of rat renal allografts. *Transplantation* **56**, 643-650 (1993).
181. Russell,P.S., Chase,C.M. & Colvin,R.B. Alloantibody- and T cell-mediated immunity in the pathogenesis of transplant arteriosclerosis: lack of progression to sclerotic lesions in B cell-deficient mice. *Transplantation* **64**, 1531-1536 (1997).
182. Tullius,S.G. & Tilney,N.L. Both alloantigen-dependent and -independent factors influence chronic allograft rejection. *Transplantation* **59**, 313-318 (1995).
183. Azuma,H., Tullius,S.G., Heemann,U.W. & Tilney,N.L. Nonimmune factors may contribute to chronic rejection of kidney transplants. *Transplant. Proc.* **26**, 2109-2110 (1994).

184. Cecka, J.M. Early rejection: determining the fate of renal transplants. *Transplant. Proc.* **23**, 1263-1264 (1991).
185. Jindal, R.M. & Hariharan, S. Chronic rejection in kidney transplants. An in-depth review. *Nephron* **83**, 13-24 (1999).
186. Tesi, R.J. *et al.* Acute rejection episodes: best predictor of long-term primary cadaveric renal transplant survival. *Transplant. Proc.* **25**, 901-902 (1993).
187. Basadonna, G.P. *et al.* Early versus late acute renal allograft rejection: impact on chronic rejection. *Transplantation* **55**, 993-995 (1993).
188. Massy, Z.A., Guijarro, C., Wiederkehr, M.R., Ma, J.Z. & Kasiske, B.L. Chronic renal allograft rejection: immunologic and nonimmunologic risk factors. *Kidney Int.* **49**, 518-524 (1996).
189. Montagnino, G. *et al.* Prognostic factors of long-term allograft survival in 632 CyA-treated recipients of a primary renal transplant. *Transpl. Int.* **10**, 268-275 (1997).
190. Yilmaz, S. & Hayry, P. The impact of acute episodes of rejection on the generation of chronic rejection in rat renal allografts. *Transplantation* **56**, 1153-1156 (1993).
191. Tejani, A., Cortes, L. & Stablein, D. Clinical correlates of chronic rejection in pediatric renal transplantation. A report of the North American Pediatric Renal Transplant Cooperative Study. *Transplantation* **61**, 1054-1058 (1996).
192. van Saase, J.L. *et al.* The relation between acute vascular and interstitial renal allograft rejection and subsequent chronic rejection. *Transplantation* **59**, 1280-1285 (1995).
193. Opelz, G. HLA matching should be utilized for improving kidney transplant success rates. *Transplant. Proc.* **23**, 46-50 (1991).
194. Opelz, G. *et al.* Long-term impact of HLA matching on kidney graft survival in cyclosporine-treated recipients. *Transplant. Proc.* **23**, 373-375 (1991).
195. Opelz, G. *et al.* Survival of DNA HLA-DR typed and matched cadaver kidney transplants. The Collaborative Transplant Study. *Lancet* **338**, 461-463 (1991).
196. Morris, P.J., Johnson, R.J., Fuggle, S.V., Belger, M.A. & Briggs, J.D. Analysis of factors that affect outcome of primary cadaveric renal transplantation in the UK. HLA Task Force of the Kidney Advisory Group of the United Kingdom Transplant Support Service Authority (UKTSSA). *Lancet* **354**, 1147-1152 (1999).

197. Tejani,A. & Sullivan,E.K. Do six-antigen-matched cadaver donor kidneys provide better graft survival to children compared with one-haploidentical living-related donor transplants? A report of the North American Pediatric Renal Transplant Cooperative Study. *Pediatr. Transplant.* **4**, 140-145 (2000).
198. Terasaki,P.I., Gjertson,D.W., Cecka,J.M. & Takemoto,S. Proposed HLA matching scheme for improved cadaveric kidney allocation. *Transplant. Proc.* **27**, 61-63 (1995).
199. Legendre,C. *et al.* Histologic features of chronic allograft nephropathy revealed by protocol biopsies in kidney transplant recipients. *Transplantation* **65**, 1506-1509 (1998).
200. Kaufman,D.B., Sutherland,D.E., Noreen,H., Najarian,J.S. & Fryd,D.S. Renal transplantation between living-related sibling pairs matched for zero-HLA haplotypes. *Transplantation* **47**, 113-119 (1989).
201. Glasscock,R.J., Feldman,D., Reynolds,E.S., Dammin,G.J. & Merrill,J.P. Human renal isografts: a clinical and pathologic analysis. *Medicine (Baltimore)* **47**, 411-454 (1968).
202. Tullius,S.G., Heemann,U.W., Azuma,H., Hancock,W.W. & Tilney,N.L. Alloantigen-independent factors lead to signs of chronic rejection in long-term kidney isografts. *Transpl. Int.* **7 Suppl 1**, S306-S307 (1994).
203. Tullius,S.G. *et al.* Antigen-independent events mimic characteristic changes of chronic allograft rejection. *Transplant. Proc.* **27**, 561-563 (1995).
204. Connolly,J.K. *et al.* Importance of minimizing HLA-DR mismatch and cold preservation time in cadaveric renal transplantation. *Transplantation* **61**, 709-714 (1996).
205. Wanders,A. *et al.* Ischemia-induced transplant arteriosclerosis in the rat. *Arterioscler. Thromb. Vasc. Biol.* **15**, 145-155 (1995).
206. Yuge,J. & Cecka,J.M. Sex and age effects in renal transplantation. *Clin. Transpl.* 257-267 (1991).
207. Pratschke,J. *et al.* Accelerated rejection of renal allografts from brain-dead donors. *Ann. Surg.* **232**, 263-271 (2000).
208. Okamoto,S. *et al.* Impact of brain death on hormonal homeostasis and hepatic microcirculation of transplant organ donors. *Transpl. Int.* **11 Suppl 1**, S404-S407 (1998).
209. Kusaka,M. *et al.* Activation of inflammatory mediators in rat renal isografts by donor brain death. *Transplantation* **69**, 405-410 (2000).



210. Lagiewska,B., Pacholczyk,M., Szostek,M., Walaszewski,J. & Rowinski,W. Hemodynamic and metabolic disturbances observed in brain-dead organ donors. *Transplant. Proc.* **28**, 165-166 (1996).
211. Nagareda,T. *et al.* Clinicopathology of kidneys from brain-dead patients treated with vasopressin and epinephrine. *Kidney Int.* **43**, 1363-1370 (1993).
212. Shoskes,D.A. & Cecka,J.M. Deleterious effects of delayed graft function in cadaveric renal transplant recipients independent of acute rejection. *Transplantation* **66**, 1697-1701 (1998).
213. Tullius,S.G., Heemann,U., Azuma,H., Pradham,P. & Tilney,N.L. Early ischemic injury leads to long-term functional and morphologic deterioration of naive rat kidneys and may contribute to changes of chronic allograft rejection. *Transplant. Proc.* **26**, 2041-2042 (1994).
214. Gjertson,D.W. Impact of delayed graft function and acute rejection on kidney graft survival. *Clin. Transpl.* 467-480 (2000).
215. Fellstrom,B. *et al.* The relative influence of antigen-dependent and independent factors for development and progression of chronic rejection. *Transplant. Proc.* **31**, 2717-2718 (1999).
216. Herrero-Fresneda,I. *et al.* Do alloreactivity and prolonged cold ischemia cause different elementary lesions in chronic allograft nephropathy? *Am. J. Pathol.* **162**, 127-137 (2003).
217. Sagedal,S. *et al.* Impact of early cytomegalovirus infection and disease on long-term recipient and kidney graft survival. *Kidney Int.* **66**, 329-337 (2004).
218. Opelz,G., Wujciak,T. & Ritz,E. Association of chronic kidney graft failure with recipient blood pressure. Collaborative Transplant Study. *Kidney Int.* **53**, 217-222 (1998).
219. Fellstrom,B. The effects of lipids on graft outcome. *Transplant. Proc.* **31**, 14S-15S (1999).
220. Dimeny,E. *et al.* The influence of pretransplant lipoprotein abnormalities on the early results of renal transplantation. *Eur. J. Clin. Invest* **23**, 572-579 (1993).
221. Kasiske,B.L., Kalil,R.S., Lee,H.S. & Rao,K.V. Histopathologic findings associated with a chronic, progressive decline in renal allograft function. *Kidney Int.* **40**, 514-524 (1991).
222. Leonarduzzi,G. *et al.* Up-regulation of the fibrogenic cytokine TGF-beta1 by oxysterols: a mechanistic link between cholesterol and atherosclerosis. *FASEB J.* **15**, 1619-1621 (2001).

223. Fine,L.G., Norman,J.T. & Ong,A. Cell-cell cross-talk in the pathogenesis of renal interstitial fibrosis. *Kidney Int. Suppl* **49**, S48-S50 (1995).
224. Abo-Zenah,H. *et al.* Early progressive interstitial fibrosis in human renal allografts. *Clin. Nephrol.* **57**, 9-18 (2002).
225. Hume,D.M., Merrill,J.P., Miller,B.F. & Thorn,G.W. Experiences with renal homotransplantation in the human: report of nine cases. *J. Clin. Invest* **34**, 327-382 (1955).
226. Solez,K. *et al.* International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. *Kidney Int.* **44**, 411-422 (1993).
227. Racusen,L.C. *et al.* The Banff 97 working classification of renal allograft pathology. *Kidney Int.* **55**, 713-723 (1999).
228. Fellstrom,B. Immune injury--is it all there is to chronic graft rejection? *Nephrol. Dial. Transplant.* **10**, 149-151 (1995).
229. Tullius,S.G. *et al.* Binding of lymphocytes to acutely rejecting rat kidney allografts in vitro is guided by events in the graft itself rather than by sensitization of host lymphocytes. *Transpl. Immunol.* **3**, 91-97 (1995).
230. Lemstrom,K., Koskinen,P. & Hayry,P. Molecular mechanisms of chronic renal allograft rejection. *Kidney Int. Suppl* **52**, S2-10 (1995).
231. Russell,M.E. *et al.* Upregulation of cytokines associated with macrophage activation in the Lewis-to-F344 rat transplantation model of chronic cardiac rejection. *Transplantation* **59**, 572-578 (1995).
232. Russell,M.E. Macrophages and transplant arteriosclerosis: known and novel molecules. *J. Heart Lung Transplant.* **14**, S111-S115 (1995).
233. Sasaguri,S. *et al.* Role of smooth-muscle cells and macrophages in cardiac allograft arteriosclerosis in rabbits. *J. Heart Transplant.* **9**, 18-24 (1990).
234. Demetris,A.J., Zerbe,T. & Banner,B. Morphology of solid organ allograft arteriopathy: identification of proliferating intimal cell populations. *Transplant. Proc.* **21**, 3667-3669 (1989).
235. Nicleleit,V., Vamvakas,E.C., Pascual,M., Poletti,B.J. & Colvin,R.B. The prognostic significance of specific arterial lesions in acute renal allograft rejection. *J. Am. Soc. Nephrol.* **9**, 1301-1308 (1998).
236. Colvin,R.B. The renal allograft biopsy. *Kidney Int.* **50**, 1069-1082 (1996).
237. Hayry,P. *et al.* Cellular and molecular mechanisms in allograft arteriosclerosis. *Transplant. Proc.* **24**, 2359-2361 (1992).

238. Barrientos,A. *et al.* Glomerular hyperfiltration as a nonimmunologic mechanism of progression of chronic renal rejection. *Transplantation* **57**, 753-756 (1994).
239. Habib,R. *et al.* A specific glomerular lesion of the graft: allograft glomerulopathy. *Kidney Int. Suppl* **42**, S104-S111 (1993).
240. Cheigh,J.S. *et al.* Focal segmental glomerulosclerosis in renal transplants. *Am. J. Kidney Dis.* **2**, 449-455 (1983).
241. Croker,B.P., Clapp,W.L., Abu Shamat,A.R., Kone,B.C. & Peterson,J.C. Macrophages and chronic renal allograft nephropathy. *Kidney Int. Suppl* **57**, S42-S49 (1996).
242. Goto,E. *et al.* Mast cells in human allografted kidney: correlation with interstitial fibrosis. *Clin. Transplant.* **16 Suppl 8**, 7-11 (2002).
243. Akalin,E., Dikman,S., Murphy,B., Bromberg,J.S. & Hancock,W.W. Glomerular infiltration by CXCR3+ ICOS+ activated T cells in chronic allograft nephropathy with transplant glomerulopathy. *Am. J. Transplant.* **3**, 1116-1120 (2003).
244. Hamar,P., Szabo,A., Muller,V. & Heemann,U. The involvement of activated T cells and growth-factor production in the early and late phase of chronic kidney allograft nephropathy in rats. *Transpl. Int.* **15**, 446-454 (2002).
245. Song,E. *et al.* Early application of Met-RANTES ameliorates chronic allograft nephropathy. *Kidney Int.* **61**, 676-685 (2002).
246. Mason,D.W. & Morris,P.J. Effector mechanisms in allograft rejection. *Annu. Rev. Immunol.* **4**, 119-145 (1986).
247. Heemann,U.W. *et al.* Infiltration patterns of macrophages and lymphocytes in chronically rejecting rat kidney allografts. *Transpl. Int.* **7**, 349-355 (1994).
248. Pilmore,H.L., Painter,D.M., Bishop,G.A., McCaughan,G.W. & Eris,J.M. Early up-regulation of macrophages and myofibroblasts: a new marker for development of chronic renal allograft rejection. *Transplantation* **69**, 2658-2662 (2000).
249. Bogman,M.J. *et al.* Diagnosis of renal allograft rejection by macrophage immunostaining with a CD14 monoclonal antibody, WT14. *Lancet* **2**, 235-238 (1989).
250. Von Willebrand,E. & Hayry,P. Composition and in vitro cytotoxicity of cellular infiltrates in rejecting human kidney allografts. *Cell Immunol.* **41**, 358-372 (1978).

251. Hancock, W.W., Thomson, N.M. & Atkins, R.C. Composition of interstitial cellular infiltrate identified by monoclonal antibodies in renal biopsies of rejecting human renal allografts. *Transplantation* **35**, 458-463 (1983).
252. Ozdemir, B.H., Demirhan, B. & Gungen, Y. The presence and prognostic importance of glomerular macrophage infiltration in renal allografts. *Nephron* **90**, 442-446 (2002).
253. Dooper, I.M. *et al.* The extent of peritubular CD14 staining in renal allografts as an independent immunohistological marker for acute rejection. *Transplantation* **58**, 820-827 (1994).
254. Yanagisawa, T. *et al.* Relationship between macrophage infiltration of renal allografts and chronic renal impairment. *Transplant. Proc.* **29**, 2783-2786 (1997).
255. Nadeau, K.C., Azuma, H. & Tilney, N.L. Sequential cytokine dynamics in chronic rejection of rat renal allografts: roles for cytokines RANTES and MCP-1. *Proc. Natl. Acad. Sci. U. S. A* **92**, 8729-8733 (1995).
256. Adams, D.H., Wyner, L.R. & Karnovsky, M.J. Experimental graft arteriosclerosis. II. Immunocytochemical analysis of lesion development. *Transplantation* **56**, 794-799 (1993).
257. Desmouliere, A. Factors influencing myofibroblast differentiation during wound healing and fibrosis. *Cell Biol. Int.* **19**, 471-476 (1995).
258. Ishibashi, M. *et al.* A gamma-lactone immunosuppressant inhibits the generation of macrophage killer cells and prolongs skin allograft survival in the rat. *Transplant. Proc.* **24**, 1411-1412 (1992).
259. Loong, C.C., Chen, A., Lui, W.Y., King, K.L. & Lin, C.Y. Expression of cytokines, growth factors, and adhesion molecules in rejecting human renal allograft. *Transplant. Proc.* **28**, 1445-1446 (1996).
260. Ruster, M., Sperschneider, H., Funfstuck, R., Stein, G. & Grone, H.J. Differential expression of beta-chemokines MCP-1 and RANTES and their receptors CCR1, CCR2, CCR5 in acute rejection and chronic allograft nephropathy of human renal allografts. *Clin. Nephrol.* **61**, 30-39 (2004).
261. Ross, R. *et al.* Localization of PDGF-B protein in macrophages in all phases of atherogenesis. *Science* **248**, 1009-1012 (1990).
262. Savikko, J., Taskinen, E. & Von Willebrand, E. Chronic allograft nephropathy is prevented by inhibition of platelet-derived growth factor receptor: tyrosine kinase inhibitors as a potential therapy. *Transplantation* **75**, 1147-1153 (2003).

263. Lindner,V., Lappi,D.A., Baird,A., Majack,R.A. & Reidy,M.A. Role of basic fibroblast growth factor in vascular lesion formation. *Circ. Res.* **68**, 106-113 (1991).
264. Kerby,J.D. *et al.* Immunolocalization of FGF-1 and receptors in glomerular lesions associated with chronic human renal allograft rejection. *Transplantation* **62**, 190-200 (1996).
265. Pilmore,H.L., Eris,J.M., Painter,D.M., Bishop,G.A. & McCaughan,G.W. Vascular endothelial growth factor expression in human chronic renal allograft rejection. *Transplantation* **67**, 929-933 (1999).
266. Grone,H.J., Simon,M. & Grone,E.F. Expression of vascular endothelial growth factor in renal vascular disease and renal allografts. *J. Pathol.* **177**, 259-267 (1995).
267. Wakelin,S.J. *et al.* The role of vascular endothelial growth factor in the kidney in health and disease. *Nephron Physiol* **98**, 73-79 (2004).
268. Suthanthiran,M. Molecular analyses of human renal allografts: differential intragraft gene expression during rejection. *Kidney Int. Suppl* **58**, S15-S21 (1997).
269. Shihab,F.S., Tanner,A.M., Shao,Y. & Weffer,M.I. Expression of TGF-beta 1 and matrix proteins is elevated in rats with chronic rejection. *Kidney Int.* **50**, 1904-1913 (1996).
270. Nicholson,M.L. *et al.* Intragraft expression of transforming growth factor beta1 gene in isolated glomeruli from human renal transplants. *Br. J. Surg.* **86**, 1144-1148 (1999).
271. Terrell,T.G., Working,P.K., Chow,C.P. & Green,J.D. Pathology of recombinant human transforming growth factor-beta 1 in rats and rabbits. *Int. Rev. Exp. Pathol.* **34 Pt B**, 43-67 (1993).
272. Cuhaci,B. *et al.* Transforming growth factor-beta levels in human allograft chronic fibrosis correlate with rate of decline in renal function. *Transplantation* **68**, 785-790 (1999).
273. Zeisberg,M. *et al.* Renal fibrosis: collagen composition and assembly regulates epithelial-mesenchymal transdifferentiation. *Am. J. Pathol.* **159**, 1313-1321 (2001).
274. Igotz,R.A., Endo,T. & Massague,J. Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-beta. *J. Biol. Chem.* **262**, 6443-6446 (1987).
275. Wahl,S.M. Transforming growth factor beta (TGF-beta) in inflammation: a cause and a cure. *J. Clin. Immunol.* **12**, 61-74 (1992).

276. Laiho,M., Saksela,O. & Keski-Oja,J. Transforming growth factor-beta induction of type-1 plasminogen activator inhibitor. Pericellular deposition and sensitivity to exogenous urokinase. *J. Biol. Chem.* **262**, 17467-17474 (1987).
277. Roberts,A.B. & Sporn,M.B. Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). *Growth Factors* **8**, 1-9 (1993).
278. Beck,L.S. *et al.* One systemic administration of transforming growth factor-beta 1 reverses age- or glucocorticoid-impaired wound healing. *J. Clin. Invest* **92**, 2841-2849 (1993).
279. Zugmaier,G. *et al.* Transforming growth factor beta 1 induces cachexia and systemic fibrosis without an antitumor effect in nude mice. *Cancer Res.* **51**, 3590-3594 (1991).
280. Battegay,E.J., Raines,E.W., Seifert,R.A., Bowen-Pope,D.F. & Ross,R. TGF-beta induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. *Cell* **63**, 515-524 (1990).
281. Pepper,M.S., Belin,D., Montesano,R., Orci,L. & Vassalli,J.D. Transforming growth factor-beta 1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells in vitro. *J. Cell Biol.* **111**, 743-755 (1990).
282. Fan,J.M. *et al.* Transforming growth factor-beta regulates tubular epithelial-myofibroblast transdifferentiation in vitro. *Kidney Int.* **56**, 1455-1467 (1999).
283. Brandes,M.E., Allen,J.B., Ogawa,Y. & Wahl,S.M. Transforming growth factor beta 1 suppresses acute and chronic arthritis in experimental animals. *J. Clin. Invest* **87**, 1108-1113 (1991).
284. Obberghen-Schilling,E., Roche,N.S., Flanders,K.C., Sporn,M.B. & Roberts,A.B. Transforming growth factor beta 1 positively regulates its own expression in normal and transformed cells. *J. Biol. Chem.* **263**, 7741-7746 (1988).
285. Khanna,A., Cairns,V. & Hosenpud,J.D. Tacrolimus induces increased expression of transforming growth factor-beta1 in mammalian lymphoid as well as nonlymphoid cells. *Transplantation* **67**, 614-619 (1999).
286. Saggi,S.J., Andoh,T.F., Safirstein,R. & Bennett,W.M. Cyclosporin induces renal proto-oncogene RNA message and increased transforming growth factor-beta prior to renal fibrosis: Modification by calcium channel blockade in the salt replete rat. *Nephrology. (Carlton.)* **9**, 58-64 (2004).
287. Gordon,S. Alternative activation of macrophages. *Nat. Rev. Immunol.* **3**, 23-35 (2003).



288. Goerdts, S. *et al.* Alternative versus classical activation of macrophages. *Pathobiology* **67**, 222-226 (1999).
289. Erwig, L.P. & Rees, A.J. Macrophage activation and programming and its role for macrophage function in glomerular inflammation. *Kidney Blood Press Res.* **22**, 21-25 (1999).
290. Kielian, T.L. & Blecha, F. CD14 and other recognition molecules for lipopolysaccharide: a review. *Immunopharmacology* **29**, 187-205 (1995).
291. Takeda, K., Kaisho, T. & Akira, S. Toll-like receptors. *Annu. Rev. Immunol.* **21**, 335-376 (2003).
292. Janeway, C.A., Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* **54 Pt 1**, 1-13 (1989).
293. Krieger, M. & Herz, J. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem.* **63**, 601-637 (1994).
294. Peiser, L., Mukhopadhyay, S. & Gordon, S. Scavenger receptors in innate immunity. *Curr. Opin. Immunol.* **14**, 123-128 (2002).
295. Krieger, M. & Stern, D.M. Series introduction: multiligand receptors and human disease. *J. Clin. Invest* **108**, 645-647 (2001).
296. Geijtenbeek, T.B., Engering, A. & van Kooyk, Y. DC-SIGN, a C-type lectin on dendritic cells that unveils many aspects of dendritic cell biology. *J. Leukoc. Biol.* **71**, 921-931 (2002).
297. Zamze, S. *et al.* Recognition of bacterial capsular polysaccharides and lipopolysaccharides by the macrophage mannose receptor. *J. Biol. Chem.* **277**, 41613-41623 (2002).
298. Beutler, B. & Rietschel, E.T. Innate immune sensing and its roots: the story of endotoxin. *Nat. Rev. Immunol.* **3**, 169-176 (2003).
299. Sabroe, I. *et al.* Toll-like receptors in health and disease: complex questions remain. *J. Immunol.* **171**, 1630-1635 (2003).
300. Medzhitov, R. Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* **1**, 135-145 (2001).
301. Medzhitov, R., Preston-Hurlburt, P. & Janeway, C.A., Jr. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* **388**, 394-397 (1997).
302. Ulevitch, R.J. & Tobias, P.S. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu. Rev. Immunol.* **13**, 437-457 (1995).

303. Bausinger,H. *et al.* Endotoxin-free heat-shock protein 70 fails to induce APC activation. *Eur. J. Immunol.* **32**, 3708-3713 (2002).
304. Lin,G. *et al.* Regulation of interleukin-8 expression in porcine alveolar macrophages by bacterial lipopolysaccharide. *J. Biol. Chem.* **269**, 77-85 (1994).
305. Akira,S., Takeda,K. & Kaisho,T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* **2**, 675-680 (2001).
306. Brightbill,H.D. *et al.* Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science* **285**, 732-736 (1999).
307. Yoshimura,A. *et al.* Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J. Immunol.* **163**, 1-5 (1999).
308. Opitz,B. *et al.* Toll-like receptor-2 mediates Treponema glycolipid and lipoteichoic acid-induced NF-kappaB translocation. *J. Biol. Chem.* **276**, 22041-22047 (2001).
309. Alexopoulou,L., Holt,A.C., Medzhitov,R. & Flavell,R.A. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**, 732-738 (2001).
310. Hayashi,F. *et al.* The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**, 1099-1103 (2001).
311. Okamura,Y. *et al.* The extra domain A of fibronectin activates Toll-like receptor 4. *J. Biol. Chem.* **276**, 10229-10233 (2001).
312. Termeer,C. *et al.* Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J. Exp. Med.* **195**, 99-111 (2002).
313. Johnson,G.B., Brunn,G.J., Kodaira,Y. & Platt,J.L. Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. *J. Immunol.* **168**, 5233-5239 (2002).
314. Smiley,S.T., King,J.A. & Hancock,W.W. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J. Immunol.* **167**, 2887-2894 (2001).
315. Guillot,L. *et al.* Cutting edge: the immunostimulatory activity of the lung surfactant protein-A involves Toll-like receptor 4. *J. Immunol.* **168**, 5989-5992 (2002).
316. Biragyn,A. *et al.* Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science* **298**, 1025-1029 (2002).

317. Vabulas,R.M. *et al.* Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *J. Biol. Chem.* **276**, 31332-31339 (2001).
318. Ohashi,K., Burkart,V., Flohe,S. & Kolb,H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J. Immunol.* **164**, 558-561 (2000).
319. Sasu,S., LaVerda,D., Qureshi,N., Golenbock,D.T. & Beasley,D. Chlamydia pneumoniae and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. *Circ. Res.* **89**, 244-250 (2001).
320. Bulut,Y. *et al.* Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. *J. Immunol.* **168**, 1435-1440 (2002).
321. Kol,A., Bourcier,T., Lichtman,A.H. & Libby,P. Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. *J. Clin. Invest* **103**, 571-577 (1999).
322. Vabulas,R.M. *et al.* HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *J. Biol. Chem.* **277**, 15107-15112 (2002).
323. Asea,A. *et al.* HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat. Med.* **6**, 435-442 (2000).
324. Dybdahl,B. *et al.* Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through toll-like receptor-4. *Circulation* **105**, 685-690 (2002).
325. Srivastava,P. Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annu. Rev. Immunol.* **20**, 395-425 (2002).
326. Gallucci,S., Lolkema,M. & Matzinger,P. Natural adjuvants: endogenous activators of dendritic cells. *Nat. Med.* **5**, 1249-1255 (1999).
327. Matzinger,P. An innate sense of danger. *Semin. Immunol.* **10**, 399-415 (1998).
328. Gao,B. & Tsan,M.F. Recombinant human heat shock protein 60 does not induce the release of tumor necrosis factor alpha from murine macrophages. *J. Biol. Chem.* **278**, 22523-22529 (2003).
329. Gao,B. & Tsan,M.F. Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages. *J. Biol. Chem.* **278**, 174-179 (2003).

330. Medzhitov,R. *et al.* MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol. Cell* **2**, 253-258 (1998).
331. Kawai,T., Adachi,O., Ogawa,T., Takeda,K. & Akira,S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity*. **11**, 115-122 (1999).
332. Muzio,M., Ni,J., Feng,P. & Dixit,V.M. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* **278**, 1612-1615 (1997).
333. Muzio,M., Natoli,G., Saccani,S., Levrero,M. & Mantovani,A. The human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J. Exp. Med.* **187**, 2097-2101 (1998).
334. Wesche,H., Henzel,W.J., Shillinglaw,W., Li,S. & Cao,Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity*. **7**, 837-847 (1997).
335. Kaisho,T., Takeuchi,O., Kawai,T., Hoshino,K. & Akira,S. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J. Immunol.* **166**, 5688-5694 (2001).
336. Kaisho,T. & Akira,S. Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. *Trends Immunol.* **22**, 78-83 (2001).
337. Horng,T., Barton,G.M. & Medzhitov,R. TIRAP: an adapter molecule in the Toll signaling pathway. *Nat. Immunol.* **2**, 835-841 (2001).
338. Fitzgerald,K.A. *et al.* Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature* **413**, 78-83 (2001).
339. Burns,K. *et al.* Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. *Nat. Cell Biol.* **2**, 346-351 (2000).
340. Zhang,G. & Ghosh,S. Negative regulation of toll-like receptor-mediated signaling by Tollip. *J. Biol. Chem.* **277**, 7059-7065 (2002).
341. May,M.J. & Ghosh,S. Signal transduction through NF-kappa B. *Immunol. Today* **19**, 80-88 (1998).
342. Guha,M. & Mackman,N. LPS induction of gene expression in human monocytes. *Cell Signal.* **13**, 85-94 (2001).
343. Shoham,S., Huang,C., Chen,J.M., Golenbock,D.T. & Levitz,S.M. Toll-like receptor 4 mediates intracellular signaling without TNF-alpha release in response to *Cryptococcus neoformans* polysaccharide capsule. *J. Immunol.* **166**, 4620-4626 (2001).

344. Hirschfeld,M. *et al.* Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. *Infect. Immun.* **69**, 1477-1482 (2001).
345. Toshchakov,V. *et al.* TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat. Immunol.* **3**, 392-398 (2002).
346. Jones,B.W., Heldwein,K.A., Means,T.K., Saukkonen,J.J. & Fenton,M.J. Differential roles of Toll-like receptors in the elicitation of proinflammatory responses by macrophages. *Ann. Rheum. Dis.* **60 Suppl 3**, iii6-12 (2001).
347. Schilling,D., Thomas,K., Nixdorff,K., Vogel,S.N. & Fenton,M.J. Toll-like receptor 4 and Toll-IL-1 receptor domain-containing adapter protein (TIRAP)/myeloid differentiation protein 88 adapter-like (Mal) contribute to maximal IL-6 expression in macrophages. *J. Immunol.* **169**, 5874-5880 (2002).
348. Sato,S. *et al.* Synergy and cross-tolerance between toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. *J. Immunol.* **165**, 7096-7101 (2000).
349. Zhang,H., Peterson,J.W., Niesel,D.W. & Klimpel,G.R. Bacterial lipoprotein and lipopolysaccharide act synergistically to induce lethal shock and proinflammatory cytokine production. *J. Immunol.* **159**, 4868-4878 (1997).
350. Fan,J., Frey,R.S. & Malik,A.B. TLR4 signaling induces TLR2 expression in endothelial cells via neutrophil NADPH oxidase. *J. Clin. Invest* **112**, 1234-1243 (2003).
351. Kluth,D.C., Erwig,L.P. & Rees,A.J. Multiple facets of macrophages in renal injury. *Kidney Int.* **66**, 542-557 (2004).
352. Mosser,D.M. The many faces of macrophage activation. *J. Leukoc. Biol.* **73**, 209-212 (2003).
353. Stein,M., Keshav,S., Harris,N. & Gordon,S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med.* **176**, 287-292 (1992).
354. Doyle,A.G. *et al.* Interleukin-13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-gamma. *Eur. J. Immunol.* **24**, 1441-1445 (1994).
355. Jakubzick,C. *et al.* Impact of interleukin-13 responsiveness on the synthetic and proliferative properties of Th1- and Th2-type pulmonary granuloma fibroblasts. *Am. J. Pathol.* **162**, 1475-1486 (2003).

356. Anderson,C.F. & Mosser,D.M. A novel phenotype for an activated macrophage: the type 2 activated macrophage. *J. Leukoc. Biol.* **72**, 101-106 (2002).
357. Gowans,J.L. The recirculation of lymphocytes from blood to lymph in the rat. *J. Physiol* **146**, 54-69 (1959).
358. Butcher,E.C. & Picker,L.J. Lymphocyte homing and homeostasis. *Science* **272**, 60-66 (1996).
359. Larsen,C.P. *et al.* Migration and maturation of Langerhans cells in skin transplants and explants. *J. Exp. Med.* **172**, 1483-1493 (1990).
360. Reis e Sousa, Stahl,P.D. & Austyn,J.M. Phagocytosis of antigens by Langerhans cells in vitro. *J. Exp. Med.* **178**, 509-519 (1993).
361. Steinman,R.M. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9**, 271-296 (1991).
362. Pfeiffer,C. *et al.* Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. *J. Exp. Med.* **181**, 1569-1574 (1995).
363. Cramer,D.V. *et al.* Accelerated graft arteriosclerosis is enhanced by sensitization of the recipient to donor lymphocytes. *Transplant. Proc.* **21**, 3714-3715 (1989).
364. Harding,C.V. & Unanue,E.R. Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation. *Nature* **346**, 574-576 (1990).
365. Demotz,S., Grey,H.M. & Sette,A. The minimal number of class II MHC-antigen complexes needed for T cell activation. *Science* **249**, 1028-1030 (1990).
366. Reay,P.A. *et al.* Determination of the relationship between T cell responsiveness and the number of MHC-peptide complexes using specific monoclonal antibodies. *J. Immunol.* **164**, 5626-5634 (2000).
367. Irvine,D.J., Purbhoo,M.A., Krogsaard,M. & Davis,M.M. Direct observation of ligand recognition by T cells. *Nature* **419**, 845-849 (2002).
368. Hedrick,S.M., Cohen,D.I., Nielsen,E.A. & Davis,M.M. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature* **308**, 149-153 (1984).
369. Samelson,L.E. Signal transduction mediated by the T cell antigen receptor: the role of adapter proteins. *Annu. Rev. Immunol.* **20**, 371-394 (2002).



370. Harding,F.A., McArthur,J.G., Gross,J.A., Raulet,D.H. & Allison,J.P. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* **356**, 607-609 (1992).
371. McAdam,A.J., Schweitzer,A.N. & Sharpe,A.H. The role of B7 co-stimulation in activation and differentiation of CD4+ and CD8+ T cells. *Immunol. Rev.* **165**, 231-247 (1998).
372. Jenkins,M.K., Taylor,P.S., Norton,S.D. & Urdahl,K.B. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol.* **147**, 2461-2466 (1991).
373. Sharpe,A.H. & Freeman,G.J. The B7-CD28 superfamily. *Nat. Rev. Immunol.* **2**, 116-126 (2002).
374. Gross,J.A., Callas,E. & Allison,J.P. Identification and distribution of the costimulatory receptor CD28 in the mouse. *J. Immunol.* **149**, 380-388 (1992).
375. Acuto,O. & Michel,F. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat. Rev. Immunol.* **3**, 939-951 (2003).
376. June,C.H. & Riley,J.L. The CD28 Family: a T Cell Rheostat for Therapeutic Control of T Cell Activation. *Blood* (2004).
377. Ward,S.G. CD28: a signalling perspective. *Biochem. J.* **318 ( Pt 2)**, 361-377 (1996).
378. Hutchcroft,J.E. & Bierer,B.E. Activation-dependent phosphorylation of the T-lymphocyte surface receptor CD28 and associated proteins. *Proc. Natl. Acad. Sci. U. S. A* **91**, 3260-3264 (1994).
379. Viola,A. & Lanzavecchia,A. T cell activation determined by T cell receptor number and tunable thresholds. *Science* **273**, 104-106 (1996).
380. Hutloff,A. *et al.* ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* **397**, 263-266 (1999).
381. Grewal,I.S. & Flavell,R.A. CD40 and CD154 in cell-mediated immunity. *Annu. Rev. Immunol.* **16**, 111-135 (1998).
382. Mallett,S., Fossum,S. & Barclay,A.N. Characterization of the MRC OX40 antigen of activated CD4 positive T lymphocytes--a molecule related to nerve growth factor receptor. *EMBO J.* **9**, 1063-1068 (1990).
383. Paterson,D.J. *et al.* Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blasts. *Mol. Immunol.* **24**, 1281-1290 (1987).

384. Croft,M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat. Rev. Immunol.* **3**, 609-620 (2003).
385. Yashiro-Ohtani,Y. *et al.* Non-CD28 costimulatory molecules present in T cell rafts induce T cell costimulation by enhancing the association of TCR with rafts. *J. Immunol.* **164**, 1251-1259 (2000).
386. Yang,H. & Reinherz,E.L. Dynamic recruitment of human CD2 into lipid rafts. Linkage to T cell signal transduction. *J. Biol. Chem.* **276**, 18775-18785 (2001).
387. Samelson,L.E., Patel,M.D., Weissman,A.M., Harford,J.B. & Klausner,R.D. Antigen activation of murine T cells induces tyrosine phosphorylation of a polypeptide associated with the T cell antigen receptor. *Cell* **46**, 1083-1090 (1986).
388. Weiss,A., Shields,R., Newton,M., Manger,B. & Imboden,J. Ligand-receptor interactions required for commitment to the activation of the interleukin 2 gene. *J. Immunol.* **138**, 2169-2176 (1987).
389. Crabtree,G.R. Contingent genetic regulatory events in T lymphocyte activation. *Science* **243**, 355-361 (1989).
390. Monks,C.R., Freiberg,B.A., Kupfer,H., Sciaky,N. & Kupfer,A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **395**, 82-86 (1998).
391. Grakoui,A. *et al.* The immunological synapse: a molecular machine controlling T cell activation. *Science* **285**, 221-227 (1999).
392. Viola,A., Schroeder,S., Sakakibara,Y. & Lanzavecchia,A. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* **283**, 680-682 (1999).
393. Wulfig,C. & Davis,M.M. A receptor/cytoskeletal movement triggered by costimulation during T cell activation. *Science* **282**, 2266-2269 (1998).
394. Xavier,R., Brennan,T., Li,Q., McCormack,C. & Seed,B. Membrane compartmentation is required for efficient T cell activation. *Immunity.* **8**, 723-732 (1998).
395. Montixi,C. *et al.* Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. *EMBO J.* **17**, 5334-5348 (1998).
396. Janes,P.W., Ley,S.C. & Magee,A.I. Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. *J. Cell Biol.* **147**, 447-461 (1999).

397. Langlet,C., Bernard,A.M., Drevot,P. & He,H.T. Membrane rafts and signaling by the multichain immune recognition receptors. *Curr. Opin. Immunol.* **12**, 250-255 (2000).
398. Harder,T. Lipid raft domains and protein networks in T-cell receptor signal transduction. *Curr. Opin. Immunol.* **16**, 353-359 (2004).
399. Patel,V.P., Moran,M., Low,T.A. & Miceli,M.C. A molecular framework for two-step T cell signaling: Lck Src homology 3 mutations discriminate distinctly regulated lipid raft reorganization events. *J. Immunol.* **166**, 754-764 (2001).
400. Michel,F. & Acuto,O. CD28 costimulation: a source of Vav-1 for TCR signaling with the help of SLP-76? *Sci. STKE.* **2002**, E35 (2002).
401. Diehn,M. *et al.* Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. *Proc. Natl. Acad. Sci. U. S. A* **99**, 11796-11801 (2002).
402. Rincon,M. & Flavell,R.A. AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. *EMBO J.* **13**, 4370-4381 (1994).
403. Kane,L.P., Lin,J. & Weiss,A. It's all Rel-ative: NF-kappaB and CD28 costimulation of T-cell activation. *Trends Immunol.* **23**, 413-420 (2002).
404. Klee,C.B., Ren,H. & Wang,X. Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *J. Biol. Chem.* **273**, 13367-13370 (1998).
405. Xanthoudakis,S. *et al.* An enhanced immune response in mice lacking the transcription factor NFAT1. *Science* **272**, 892-895 (1996).
406. Oukka,M. *et al.* The transcription factor NFAT4 is involved in the generation and survival of T cells. *Immunity.* **9**, 295-304 (1998).
407. Alegre,M.L., Frauwirth,K.A. & Thompson,C.B. T-cell regulation by CD28 and CTLA-4. *Nat. Rev. Immunol.* **1**, 220-228 (2001).
408. Durand,D.B., Bush,M.R., Morgan,J.G., Weiss,A. & Crabtree,G.R. A 275 basepair fragment at the 5' end of the interleukin 2 gene enhances expression from a heterologous promoter in response to signals from the T cell antigen receptor. *J. Exp. Med.* **165**, 395-407 (1987).
409. Lucas,P.J., Negishi,I., Nakayama,K., Fields,L.E. & Loh,D.Y. Naive CD28-deficient T cells can initiate but not sustain an in vitro antigen-specific immune response. *J. Immunol.* **154**, 5757-5768 (1995).
410. Reichert,P., Reinhardt,R.L., Ingulli,E. & Jenkins,M.K. Cutting edge: in vivo identification of TCR redistribution and polarized IL-2 production by naive CD4 T cells. *J. Immunol.* **166**, 4278-4281 (2001).

411. Shahinian,A. *et al.* Differential T cell costimulatory requirements in CD28-deficient mice. *Science* **261**, 609-612 (1993).
412. Mosmann,T.R. & Coffman,R.L. Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv. Immunol.* **46**, 111-147 (1989).
413. Mosmann,T.R., Cherwinski,H., Bond,M.W., Giedlin,M.A. & Coffman,R.L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**, 2348-2357 (1986).
414. Ullman,K.S., Northrop,J.P., Verweij,C.L. & Crabtree,G.R. Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. *Annu. Rev. Immunol.* **8**, 421-452 (1990).
415. Snapper,C.M. & Paul,W.E. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* **236**, 944-947 (1987).
416. Parkin,J. & Cohen,B. An overview of the immune system. *Lancet* **357**, 1777-1789 (2001).
417. Mosmann,T.R. & Sad,S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today* **17**, 138-146 (1996).
418. de Waal,M.R., Abrams,J., Bennett,B., Figdor,C.G. & de Vries,J.E. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* **174**, 1209-1220 (1991).
419. Moore,K.W., O'Garra,A., de Waal,M.R., Vieira,P. & Mosmann,T.R. Interleukin-10. *Annu. Rev. Immunol.* **11**, 165-190 (1993).
420. Choi,I.S., Collisson,E.W., Maheswaran,S.K. & Yoo,H.S. Evaluation of cytokine gene expression in porcine spleen cells, peripheral blood mononuclear cells, and alveolar macrophages by competitive RT-PCR. *FEMS Immunol. Med. Microbiol.* **34**, 119-126 (2002).
421. Kurt-Jones,E.A. *et al.* Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat. Immunol.* **1**, 398-401 (2000).
422. Junqueira,L.C., Bignolas,G. & Brentani,R.R. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem. J.* **11**, 447-455 (1979).
423. Lindsay,G.K., Roslansky,P.F. & Novitsky,T.J. Single-step, chromogenic Limulus amebocyte lysate assay for endotoxin. *J. Clin. Microbiol.* **27**, 947-951 (1989).

424. Slater,T.F., Sawyer,B. & Straeuli,U. Studies on succinate-tetrazolium reductase systems. III. Points of coupling of four different tetrazolium salts. *Biochim. Biophys. Acta* **77**, 383-393 (1963).
425. Fan,H., Oro,A.E., Scott,M.P. & Khavari,P.A. Induction of basal cell carcinoma features in transgenic human skin expressing Sonic Hedgehog. *Nat. Med.* **3**, 788-792 (1997).
426. Kenney,A.M. & Rowitch,D.H. Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. *Mol. Cell Biol.* **20**, 9055-9067 (2000).
427. Roberts,D.J. *et al.* Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. *Development* **121**, 3163-3174 (1995).
428. Haraguchi,R. *et al.* Molecular analysis of external genitalia formation: the role of fibroblast growth factor (Fgf) genes during genital tubercle formation. *Development* **127**, 2471-2479 (2000).
429. Sporn,M.B., Roberts,A.B., Wakefield,L.M. & de Crombrughe,B. Some recent advances in the chemistry and biology of transforming growth factor-beta. *J. Cell Biol.* **105**, 1039-1045 (1987).
430. Edwards,D.R. *et al.* Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J.* **6**, 1899-1904 (1987).
431. Shihab,F.S. *et al.* Transforming growth factor-beta and matrix protein expression in acute and chronic rejection of human renal allografts. *J. Am. Soc. Nephrol.* **6**, 286-294 (1995).
432. Sharma,V.K. *et al.* Intragraft TGF-beta 1 mRNA: a correlate of interstitial fibrosis and chronic allograft nephropathy. *Kidney Int.* **49**, 1297-1303 (1996).
433. Noronha,I.L. *et al.* Expression of cytokines, growth factors, and their receptors in renal allograft biopsies. *Transplant. Proc.* **25**, 917-918 (1993).
434. Walgenbach,K.J., Llull,R., Murase,N., Starzl,T.E. & Hirner,A. Immunocytes of composite tissue allografts express elevated levels of TGF beta mRNA and protein during chronic rejection. *Transplant. Proc.* **29**, 1542 (1997).
435. Jain,S. *et al.* Sequential protocol biopsies from renal transplant recipients show an increasing expression of active TGF beta. *Transpl. Int.* **15**, 630-634 (2002).

436. Pilmore, H.L. *et al.* Time course of upregulation of fibrogenic growth factors and cellular infiltration in a rodent model of chronic renal allograft rejection. *Transpl. Immunol.* **10**, 245-254 (2002).
437. Ingham, P.W. & Hidalgo, A. Regulation of wingless transcription in the *Drosophila* embryo. *Development* **117**, 283-291 (1993).
438. Perrimon, N. Hedgehog and beyond. *Cell* **80**, 517-520 (1995).
439. Pertovaara, L. *et al.* Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J. Biol. Chem.* **269**, 6271-6274 (1994).
440. Renner, W. & Pilger, E. Simultaneous in vivo quantitation of vascular endothelial growth factor mRNA splice variants. *J. Vasc. Res.* **36**, 133-138 (1999).
441. Xiong, M., Elson, G., Legarda, D. & Leibovich, S.J. Production of vascular endothelial growth factor by murine macrophages: regulation by hypoxia, lactate, and the inducible nitric oxide synthase pathway. *Am. J. Pathol.* **153**, 587-598 (1998).
442. Bates, D.O. & Curry, F.E. Vascular endothelial growth factor increases microvascular permeability via a Ca(2+)-dependent pathway. *Am. J. Physiol* **273**, H687-H694 (1997).
443. Clauss, M. *et al.* Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *J. Exp. Med.* **172**, 1535-1545 (1990).
444. Barleon, B. *et al.* Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* **87**, 3336-3343 (1996).
445. Brown, L.F. *et al.* Vascular permeability factor mRNA and protein expression in human kidney. *Kidney Int.* **42**, 1457-1461 (1992).
446. Berse, B., Brown, L.F., van de, W.L., Dvorak, H.F. & Senger, D.R. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Mol. Biol. Cell* **3**, 211-220 (1992).
447. Simon, M. *et al.* Expression of vascular endothelial growth factor and its receptors in human renal ontogenesis and in adult kidney. *Am. J. Physiol* **268**, F240-F250 (1995).
448. Simon, M. *et al.* Receptors of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) in fetal and adult human kidney: localization and [125I]VEGF binding sites. *J. Am. Soc. Nephrol.* **9**, 1032-1044 (1998).



449. Kretzler, M. *et al.* Detection of multiple vascular endothelial growth factor splice isoforms in single glomerular podocytes. *Kidney Int. Suppl* **67**, S159-S161 (1998).
450. Otto, K., Duchrow, M., Broll, R., Bruch, H.P. & Strik, M.W. Expression of vascular endothelial growth factor mRNA and protein in human chronic renal allograft rejection. *Transplant. Proc.* **34**, 3134-3137 (2002).
451. Paul, L.C., Saito, K., Davidoff, A. & Benediktsson, H. Growth factor transcripts in rat renal transplants. *Am. J. Kidney Dis.* **28**, 441-450 (1996).
452. Kroshian, V.M., Sheridan, A.M. & Lieberthal, W. Functional and cytoskeletal changes induced by sublethal injury in proximal tubular epithelial cells. *Am. J. Physiol* **266**, F21-F30 (1994).
453. Pathi, S. *et al.* Comparative biological responses to human Sonic, Indian, and Desert hedgehog. *Mech. Dev.* **106**, 107-117 (2001).
454. Pons, S. & Marti, E. Sonic hedgehog synergizes with the extracellular matrix protein vitronectin to induce spinal motor neuron differentiation. *Development* **127**, 333-342 (2000).
455. Farquhar, M.G., Saito, A., Kerjaschki, D. & Orlando, R.A. The Heymann nephritis antigenic complex: megalin (gp330) and RAP. *J. Am. Soc. Nephrol.* **6**, 35-47 (1995).
456. Kerjaschki, D. & Farquhar, M.G. The pathogenic antigen of Heymann nephritis is a membrane glycoprotein of the renal proximal tubule brush border. *Proc. Natl. Acad. Sci. U. S. A* **79**, 5557-5561 (1982).
457. Moestrup, S.K. *et al.* Binding and endocytosis of proteins mediated by epithelial gp330. *Ann. N. Y. Acad. Sci.* **737**, 124-137 (1994).
458. Juhlin, C. *et al.* Monoclonal antibodies with exclusive reactivity against parathyroid cells and tubule cells of the kidney. *Proc. Natl. Acad. Sci. U. S. A* **84**, 2990-2994 (1987).
459. Argraves, W.S. Members of the low density lipoprotein receptor family control diverse physiological processes. *Front Biosci.* **6**, D406-D416 (2001).
460. Barth, J.L. & Argraves, W.S. Cubilin and megalin: partners in lipoprotein and vitamin metabolism. *Trends Cardiovasc. Med.* **11**, 26-31 (2001).
461. Willnow, T.E., Goldstein, J.L., Orth, K., Brown, M.S. & Herz, J. Low density lipoprotein receptor-related protein and gp330 bind similar ligands, including plasminogen activator-inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance. *J. Biol. Chem.* **267**, 26172-26180 (1992).

462. Kounnas,M.Z., Argraves,W.S. & Strickland,D.K. The 39-kDa receptor-associated protein interacts with two members of the low density lipoprotein receptor family, alpha 2-macroglobulin receptor and glycoprotein 330. *J. Biol. Chem.* **267**, 21162-21166 (1992).
463. Nykjaer,A. *et al.* An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. *Cell* **96**, 507-515 (1999).
464. Moestrup,S.K. *et al.* Megalin-mediated endocytosis of transcobalamin-vitamin-B12 complexes suggests a role of the receptor in vitamin-B12 homeostasis. *Proc. Natl. Acad. Sci. U. S. A* **93**, 8612-8617 (1996).
465. Birn,H. *et al.* Megalin is essential for renal proximal tubule reabsorption and accumulation of transcobalamin-B(12). *Am. J. Physiol Renal Physiol* **282**, F408-F416 (2002).
466. Marino,M., Andrews,D., Brown,D. & McCluskey,R.T. Transcytosis of retinol-binding protein across renal proximal tubule cells after megalin (gp 330)-mediated endocytosis. *J. Am. Soc. Nephrol.* **12**, 637-648 (2001).
467. Strickland,D.K., Gonias,S.L. & Argraves,W.S. Diverse roles for the LDL receptor family. *Trends Endocrinol. Metab* **13**, 66-74 (2002).
468. Beffert,U. *et al.* Reelin-mediated signaling locally regulates protein kinase B/Akt and glycogen synthase kinase 3beta. *J. Biol. Chem.* **277**, 49958-49964 (2002).
469. Gekle,M. *et al.* Transforming growth factor-beta1 reduces megalin- and cubilin-mediated endocytosis of albumin in proximal-tubule-derived opossum kidney cells. *J. Physiol* **552**, 471-481 (2003).
470. Charrier,J.B., Lapointe,F., Le Douarin,N.M. & Teillet,M.A. Anti-apoptotic role of Sonic hedgehog protein at the early stages of nervous system organogenesis. *Development* **128**, 4011-4020 (2001).
471. Fan,H. & Khavari,P.A. Sonic hedgehog opposes epithelial cell cycle arrest. *J. Cell Biol.* **147**, 71-76 (1999).
472. Qualtrough,D., Buda,A., Gaffield,W., Williams,A.C. & Paraskeva,C. Hedgehog signalling in colorectal tumour cells: induction of apoptosis with cyclopamine treatment. *Int. J. Cancer* **110**, 831-837 (2004).
473. Schwarz,C. *et al.* Failure of BCL-2 up-regulation in proximal tubular epithelial cells of donor kidney biopsy specimens is associated with apoptosis and delayed graft function. *Lab Invest* **82**, 941-948 (2002).
474. Wever,P.C. *et al.* Apoptotic tubular cell death during acute renal allograft rejection. *Clin. Nephrol.* **49**, 28-34 (1998).

475. Castaneda,M.P. *et al.* Activation of mitochondrial apoptotic pathways in human renal allografts after ischemiareperfusion injury. *Transplantation* **76**, 50-54 (2003).
476. Matzinger,P. An innate sense of danger. *Semin. Immunol.* **10**, 399-415 (1998).
477. Matzinger,P. Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* **12**, 991-1045 (1994).
478. Feder,M.E. & Hofmann,G.E. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol* **61**, 243-282 (1999).
479. Smoyer,W.E. *et al.* Ischemic acute renal failure induces differential expression of small heat shock proteins. *J. Am. Soc. Nephrol.* **11**, 211-221 (2000).
480. Wagner,M. *et al.* Heme oxygenase-1 attenuates ischemia/reperfusion-induced apoptosis and improves survival in rat renal allografts. *Kidney Int.* **63**, 1564-1573 (2003).
481. Katori,M. *et al.* Heme oxygenase-1 overexpression protects rat hearts from cold ischemia/reperfusion injury via an antiapoptotic pathway. *Transplantation* **73**, 287-292 (2002).
482. Redaelli,C.A. *et al.* Hyperthermia preconditioning induces renal heat shock protein expression, improves cold ischemia tolerance, kidney graft function and survival in rats. *Nephron* **90**, 489-497 (2002).
483. Tullius,S.G. *et al.* Induction of heme-oxygenase-1 prevents ischemia/reperfusion injury and improves long-term graft outcome in rat renal allografts. *Transplant. Proc.* **33**, 1286-1287 (2001).
484. Maulik,N. *et al.* Ischemic preconditioning reduces apoptosis by upregulating anti-death gene Bcl-2. *Circulation* **100**, II369-II375 (1999).
485. Uchiyama,T., Engelman,R.M., Maulik,N. & Das,D.K. Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. *Circulation* **109**, 3042-3049 (2004).
486. Hirai,K. *et al.* PI3K inhibition in neonatal rat brain slices during and after hypoxia reduces phospho-Akt and increases cytosolic cytochrome c and apoptosis. *Brain Res. Mol. Brain Res.* **124**, 51-61 (2004).
487. Fujio,Y., Nguyen,T., Wencker,D., Kitsis,R.N. & Walsh,K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation* **101**, 660-667 (2000).

488. Molitoris, B.A. Na(+)-K(+)-ATPase that redistributes to apical membrane during ATP depletion remains functional. *Am. J. Physiol* **265**, F693-F697 (1993).
489. Martinez, G. & Bertram, J.F. Organisation of bone morphogenetic proteins in renal development. *Nephron Exp. Nephrol.* **93**, e18-e22 (2003).
490. Simon, M. *et al.* Expression of bone morphogenetic protein-7 mRNA in normal and ischemic adult rat kidney. *Am. J. Physiol* **276**, F382-F389 (1999).
491. Dudley, A.T., Lyons, K.M. & Robertson, E.J. A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev.* **9**, 2795-2807 (1995).
492. Vukicevic, S., Kopp, J.B., Luyten, F.P. & Sampath, T.K. Induction of nephrogenic mesenchyme by osteogenic protein 1 (bone morphogenetic protein 7). *Proc. Natl. Acad. Sci. U. S. A* **93**, 9021-9026 (1996).
493. Hruska, K.A. *et al.* Osteogenic protein-1 prevents renal fibrogenesis associated with ureteral obstruction. *Am. J. Physiol Renal Physiol* **279**, F130-F143 (2000).
494. Klahr, S. The bone morphogenetic proteins (BMPs). Their role in renal fibrosis and renal function. *J. Nephrol.* **16**, 179-185 (2003).
495. Emami, A., Schwartz, J.H. & Borkan, S.C. Transient ischemia or heat stress induces a cytoprotectant protein in rat kidney. *Am. J. Physiol* **260**, F479-F485 (1991).
496. Van Why, S.K. *et al.* Induction and intracellular localization of HSP-72 after renal ischemia. *Am. J. Physiol* **263**, F769-F775 (1992).
497. Cohen, D.M., Wasserman, J.C. & Gullans, S.R. Immediate early gene and HSP70 expression in hyperosmotic stress in MDCK cells. *Am. J. Physiol* **261**, C594-C601 (1991).
498. Yuan, C.M., Bohen, E.M., Musio, F. & Carome, M.A. Sublethal heat shock and cyclosporine exposure produce tolerance against subsequent cyclosporine toxicity. *Am. J. Physiol* **271**, F571-F578 (1996).
499. Taguchi, T. *et al.* Verotoxins induce apoptosis in human renal tubular epithelium derived cells. *Kidney Int.* **53**, 1681-1688 (1998).
500. Johnson, D.W. *et al.* Cyclosporin exerts a direct fibrogenic effect on human tubulointerstitial cells: roles of insulin-like growth factor I, transforming growth factor beta1, and platelet-derived growth factor. *J. Pharmacol. Exp. Ther.* **289**, 535-542 (1999).

501. Johnson,D.W., Saunders,H.J., Field,M.J. & Pollock,C.A. In vitro effects of simvastatin on tubulointerstitial cells in a human model of cyclosporin nephrotoxicity. *Am. J. Physiol* **276**, F467-F475 (1999).
502. Yard,B.A. *et al.* CsA, FK506, corticosteroids and rapamycin inhibit TNF alpha production by cultured PTEC. *Kidney Int.* **44**, 352-358 (1993).
503. Bishop,G.A. & Hall,B.M. Effects of immunosuppressive drugs on functions of activated T lymphocytes. Cyclosporine inhibition of gamma interferon production in the presence of interleukin. *Transplantation* **45**, 967-972 (1988).
504. Lewis,E., Weiler,M., Chaimovitz,C. & Douvdevani,A. Interleukin-15 is the main mediator of lymphocyte proliferation in cultures mixed with human kidney tubular epithelial cells. *Transplantation* **72**, 886-890 (2001).
505. Kurtz,R.M. *et al.* Dexamethasone and cyclosporin A modulation of human retinal pigment epithelial cell monocyte chemotactic protein-1 and interleukin-8. *Invest Ophthalmol. Vis. Sci.* **38**, 436-445 (1997).
506. Natori,Y. *et al.* Production of monocyte chemoattractant protein-1 by cultured glomerular epithelial cells: inhibition by dexamethasone. *Exp. Nephrol.* **5**, 318-322 (1997).
507. Granucci,F., Castagnoli,P.R., Rogge,L. & Sinigaglia,F. Gene expression profiling in immune cells using microarray. *Int. Arch. Allergy Immunol.* **126**, 257-266 (2001).
508. Chtanova,T., Kemp,R.A., Sutherland,A.P., Ronchese,F. & Mackay,C.R. Gene microarrays reveal extensive differential gene expression in both CD4(+) and CD8(+) type 1 and type 2 T cells. *J. Immunol.* **167**, 3057-3063 (2001).
509. Berridge,M.V. & Tan,A.S. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch. Biochem. Biophys.* **303**, 474-482 (1993).
510. Detmer,K., Walker,A.N., Jenkins,T.M., Steele,T.A. & Dannawi,H. Erythroid differentiation in vitro is blocked by cyclopamine, an inhibitor of hedgehog signaling. *Blood Cells Mol. Dis.* **26**, 360-372 (2000).
511. Detmer,K., Steele,T.A., Shoop,M.A. & Dannawi,H. Lineage-restricted expression of bone morphogenetic protein genes in human hematopoietic cell lines. *Blood Cells Mol. Dis.* **25**, 310-323 (1999).
512. Elliget,K.A. & Trump,B.F. Primary cultures of normal rat kidney proximal tubule epithelial cells for studies of renal cell injury. *In Vitro Cell Dev. Biol.* **27A**, 739-748 (1991).

513. Trifillis,A.L., Regec,A.L. & Trump,B.F. Isolation, culture and characterization of human renal tubular cells. *J. Urol.* **133**, 324-329 (1985).
514. Taub,M. & Sato,G. Growth of functional primary cultures of kidney epithelial cells in defined medium. *J. Cell Physiol* **105**, 369-378 (1980).
515. Detrisac,C.J., Sens,M.A., Garvin,A.J., Spicer,S.S. & Sens,D.A. Tissue culture of human kidney epithelial cells of proximal tubule origin. *Kidney Int.* **25**, 383-390 (1984).
516. van den Brink,G.R. *et al.* Sonic hedgehog expression correlates with fundic gland differentiation in the adult gastrointestinal tract. *Gut* **51**, 628-633 (2002).
517. Ueda,N. & Shah,S.V. Role of intracellular calcium in hydrogen peroxide-induced renal tubular cell injury. *Am. J. Physiol* **263**, F214-F221 (1992).
518. Wang,H. & Joseph,J.A. Mechanisms of hydrogen peroxide-induced calcium dysregulation in PC12 cells. *Free Radic. Biol. Med.* **28**, 1222-1231 (2000).
519. Duchen,M.R. Mitochondria and calcium: from cell signalling to cell death. *J. Physiol* **529 Pt 1**, 57-68 (2000).
520. Ermak,G. & Davies,K.J. Calcium and oxidative stress: from cell signaling to cell death. *Mol. Immunol.* **38**, 713-721 (2002).
521. Komatsuda,A. *et al.* Overexpression of the human 72 kDa heat shock protein in renal tubular cells confers resistance against oxidative injury and cisplatin toxicity. *Nephrol. Dial. Transplant.* **14**, 1385-1390 (1999).
522. Hung,C.C., Ichimura,T., Stevens,J.L. & Bonventre,J.V. Protection of renal epithelial cells against oxidative injury by endoplasmic reticulum stress preconditioning is mediated by ERK1/2 activation. *J. Biol. Chem.* **278**, 29317-29326 (2003).
523. Cuttle,L., Zhang,X.J., Endre,Z.H., Winterford,C. & Gobe,G.C. Bcl-X(L) translocation in renal tubular epithelial cells in vitro protects distal cells from oxidative stress. *Kidney Int.* **59**, 1779-1788 (2001).
524. Szepietowski,J.C., Morita,A. & Tsuji,T. Ultraviolet B induces mast cell apoptosis: a hypothetical mechanism of ultraviolet B treatment for uraemic pruritus. *Med. Hypotheses* **58**, 167-170 (2002).
525. Mohan,S., Dhanalakshmi,S., Mallikarjuna,G.U., Singh,R.P. & Agarwal,R. Silibinin modulates UVB-induced apoptosis via mitochondrial proteins, caspases activation, and mitogen-activated protein kinase signaling in human epidermoid carcinoma A431 cells. *Biochem. Biophys. Res. Commun.* **320**, 183-189 (2004).



526. Shimmura,S., Tadano,K. & Tsubota,K. UV dose-dependent caspase activation in a corneal epithelial cell line. *Curr. Eye Res.* **28**, 85-92 (2004).
527. Andersson,E., Rosdahl,I., Torma,H. & Vahlquist,A. Differential effects of UV irradiation on nuclear retinoid receptor levels in cultured keratinocytes and melanocytes. *Exp. Dermatol.* **12**, 563-571 (2003).
528. Sethi,G. & Sodhi,A. Activation of c-Jun N-terminal kinase is required for ultraviolet B-induced apoptosis of murine peritoneal macrophages in vitro. *J. Photochem. Photobiol. B* **73**, 133-140 (2004).
529. Ding,B.X. & Wang,C.B. Inhibitory effect of polypeptides from *Chlamys farreri* on UVB-induced apoptosis and DNA damage in normal human dermal fibroblasts in vitro. *Acta Pharmacol. Sin.* **24**, 1006-1010 (2003).
530. van Kooten,C., Lam,S. & Daha,M.R. Isolation, culture, characterization and use of human renal tubular epithelial cells. *J. Nephrol.* **14**, 204-210 (2001).
531. Baer,P.C., Tunn,U.W., Nunez,G., Scherberich,J.E. & Geiger,H. Transdifferentiation of distal but not proximal tubular epithelial cells from human kidney in culture. *Exp. Nephrol.* **7**, 306-313 (1999).
532. Hammond,T.G. Analysis and isolation of renal tubular cells by flow cytometry. *Kidney Int.* **42**, 997-1005 (1992).
533. Thayer,S.P. *et al.* Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* **425**, 851-856 (2003).
534. Fujita,E. *et al.* Involvement of Sonic hedgehog in the cell growth of LK-2 cells, human lung squamous carcinoma cells. *Biochem. Biophys. Res. Commun.* **238**, 658-664 (1997).
535. Nishimaki,H. *et al.* A role of activated Sonic hedgehog signaling for the cellular proliferation of oral squamous cell carcinoma cell line. *Biochem. Biophys. Res. Commun.* **314**, 313-320 (2004).
536. Angelo,L.S., Talpaz,M. & Kurzrock,R. Autocrine interleukin-6 production in renal cell carcinoma: evidence for the involvement of p53. *Cancer Res.* **62**, 932-940 (2002).
537. Mattii,L., Bianchi,F., Da,P., I, Dolfi,A. & Bernardini,N. Renal cell cultures for the study of growth factor interactions underlying kidney organogenesis. *In Vitro Cell Dev. Biol. Anim* **37**, 251-258 (2001).
538. Yamaoka,T. *et al.* Biologic and binding activities of IFN-alpha subtypes in ACHN human renal cell carcinoma cells and Daudi Burkitt's lymphoma cells. *J. Interferon Cytokine Res.* **19**, 1343-1349 (1999).

539. Pansky,A. *et al.* Gastrin releasing peptide-preferring bombesin receptors mediate growth of human renal cell carcinoma. *J. Am. Soc. Nephrol.* **11**, 1409-1418 (2000).
540. Kahan,B.D. Cyclosporine. *N. Engl. J. Med.* **321**, 1725-1738 (1989).
541. Henderson,D.J., Naya,I., Bundick,R.V., Smith,G.M. & Schmidt,J.A. Comparison of the effects of FK-506, cyclosporin A and rapamycin on IL-2 production. *Immunology* **73**, 316-321 (1991).
542. Prashar,Y., Khanna,A., Sehajpal,P., Sharma,V.K. & Suthanthiran,M. Stimulation of transforming growth factor-beta 1 transcription by cyclosporine. *FEBS Lett.* **358**, 109-112 (1995).
543. Paslaru,L., Rallu,M., Manuel,M., Davidson,S. & Morange,M. Cyclosporin A induces an atypical heat shock response. *Biochem. Biophys. Res. Commun.* **269**, 464-469 (2000).
544. Morris,S.M., Jr., Kepka-Lenhart,D., McGill,R.L., Curthoys,N.P. & Adler,S. Specific disruption of renal function and gene transcription by cyclosporin A. *J. Biol. Chem.* **267**, 13768-13771 (1992).
545. Mitchell,D.B. & Acosta,D. Evaluation of the cytotoxicity of tricyclic antidepressants in primary cultures of rat hepatocytes. *J. Toxicol. Environ. Health* **7**, 83-92 (1981).
546. Paslaru,L., Pinto,M. & Morange,M. GRP78 induction by cyclosporin A in human HeLa cells. *FEBS Lett.* **350**, 304-308 (1994).
547. Takahashi,H. *et al.* Roxithromycin decreases ultraviolet B irradiation-induced reactive oxygen intermediates production and apoptosis of keratinocytes. *J. Dermatol. Sci.* **34**, 25-33 (2004).
548. Bornemann,D.J., Duncan,J.E., Staatz,W., Selleck,S. & Warrior,R. Abrogation of heparan sulfate synthesis in *Drosophila* disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways. *Development* **131**, 1927-1938 (2004).
549. Nakamura,T. *et al.* Induction of osteogenic differentiation by hedgehog proteins. *Biochem. Biophys. Res. Commun.* **237**, 465-469 (1997).
550. Zeng,X. *et al.* A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature* **411**, 716-720 (2001).
551. Marigo,V., Scott,M.P., Johnson,R.L., Goodrich,L.V. & Tabin,C.J. Conservation in hedgehog signaling: induction of a chicken patched homolog by Sonic hedgehog in the developing limb. *Development* **122**, 1225-1233 (1996).

552. Alcedo,J. & Noll,M. Hedgehog and its patched-smoothened receptor complex: a novel signalling mechanism at the cell surface. *Biol. Chem.* **378**, 583-590 (1997).
553. van den,H.M. & Ingham,P.W. smoothened encodes a receptor-like serpentine protein required for hedgehog signalling. *Nature* **382**, 547-551 (1996).
554. Ingham,P.W. Signalling by hedgehog family proteins in Drosophila and vertebrate development. *Curr. Opin. Genet. Dev.* **5**, 492-498 (1995).
555. Leung,D.W., Cachianes,G., Kuang,W.J., Goeddel,D.V. & Ferrara,N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* **246**, 1306-1309 (1989).
556. Keck,P.J. *et al.* Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* **246**, 1309-1312 (1989).
557. Bates,D.O. & Curry,F.E. Vascular endothelial growth factor increases hydraulic conductivity of isolated perfused microvessels. *Am. J. Physiol* **271**, H2520-H2528 (1996).
558. Danielpour,D. Improved sandwich enzyme-linked immunosorbent assays for transforming growth factor beta 1. *J. Immunol. Methods* **158**, 17-25 (1993).
559. Danielpour,D. *et al.* Sandwich enzyme-linked immunosorbent assays (SELISAs) quantitate and distinguish two forms of transforming growth factor-beta (TGF-beta 1 and TGF-beta 2) in complex biological fluids. *Growth Factors* **2**, 61-71 (1989).
560. Stoy,N. Macrophage biology and pathobiology in the evolution of immune responses: a functional analysis. *Pathobiology* **69**, 179-211 (2001).
561. Ito,T. & Kodama,M. Demonstration by reverse transcription-polymerase chain reaction of multiple cytokine mRNA expression in bovine alveolar macrophages and peripheral blood mononuclear cells. *Res. Vet. Sci.* **60**, 94-96 (1996).
562. Delahooke,D.M., Barclay,G.R. & Poxton,I.R. Tumor necrosis factor induction by an aqueous phenol-extracted lipopolysaccharide complex from Bacteroides species. *Infect. Immun.* **63**, 840-846 (1995).
563. Gardiner,J.S., Keil,L.B. & DeBari,V.A. In vitro formation of complement activation products by lipopolysaccharide chemotypes of Salmonella minnesota. *Int. Arch. Allergy Appl. Immunol.* **96**, 51-54 (1991).
564. Gallucci,S., Lolkema,M. & Matzinger,P. Natural adjuvants: endogenous activators of dendritic cells. *Nat. Med.* **5**, 1249-1255 (1999).

565. Sauter,B. *et al.* Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J. Exp. Med.* **191**, 423-434 (2000).
566. Chen,W., Syldath,U., Bellmann,K., Burkart,V. & Kolb,H. Human 60-kDa heat-shock protein: a danger signal to the innate immune system. *J. Immunol.* **162**, 3212-3219 (1999).
567. Singh-Jasuja,H. *et al.* The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. *Eur. J. Immunol.* **30**, 2211-2215 (2000).
568. Basu,S., Binder,R.J., Suto,R., Anderson,K.M. & Srivastava,P.K. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. *Int. Immunol.* **12**, 1539-1546 (2000).
569. Bethke,K. *et al.* Different efficiency of heat shock proteins (HSP) to activate human monocytes and dendritic cells: superiority of HSP60. *J. Immunol.* **169**, 6141-6148 (2002).
570. Ohashi,K., Burkart,V., Flohe,S. & Kolb,H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J. Immunol.* **164**, 558-561 (2000).
571. Panjwani,N.N., Popova,L. & Srivastava,P.K. Heat shock proteins gp96 and hsp70 activate the release of nitric oxide by APCs. *J. Immunol.* **168**, 2997-3003 (2002).
572. Medzhitov,R. & Janeway,C.A., Jr. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* **91**, 295-298 (1997).
573. Sabroe,I. *et al.* Toll-like receptors in health and disease: complex questions remain. *J. Immunol.* **171**, 1630-1635 (2003).
574. Vabulas,R.M. *et al.* Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *J. Biol. Chem.* **276**, 31332-31339 (2001).
575. Asea,A. *et al.* Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J. Biol. Chem.* **277**, 15028-15034 (2002).
576. Okamura,Y. *et al.* The extra domain A of fibronectin activates Toll-like receptor 4. *J. Biol. Chem.* **276**, 10229-10233 (2001).
577. Saito,S. *et al.* The fibronectin extra domain A activates matrix metalloproteinase gene expression by an interleukin-1-dependent mechanism. *J. Biol. Chem.* **274**, 30756-30763 (1999).

578. Termeer,C. *et al.* Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J. Exp. Med.* **195**, 99-111 (2002).
579. Johnson,G.B., Brunn,G.J., Kodaira,Y. & Platt,J.L. Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. *J. Immunol.* **168**, 5233-5239 (2002).
580. Wrenshall,L.E., Cerra,F.B., Singh,R.K. & Platt,J.L. Heparan sulfate initiates signals in murine macrophages leading to divergent biologic outcomes. *J. Immunol.* **154**, 871-880 (1995).
581. Smiley,S.T., King,J.A. & Hancock,W.W. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J. Immunol.* **167**, 2887-2894 (2001).
582. Chen,Y., Goodman,R.H. & Smolik,S.M. Cubitus interruptus requires Drosophila CREB-binding protein to activate wingless expression in the Drosophila embryo. *Mol. Cell Biol.* **20**, 1616-1625 (2000).
583. Shimazu,R. *et al.* MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med.* **189**, 1777-1782 (1999).
584. Hailman,E. *et al.* Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J. Exp. Med.* **179**, 269-277 (1994).
585. Wright,S.D., Ramos,R.A., Tobias,P.S., Ulevitch,R.J. & Mathison,J.C. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249**, 1431-1433 (1990).
586. Moroi,Y. *et al.* Induction of cellular immunity by immunization with novel hybrid peptides complexed to heat shock protein 70. *Proc. Natl. Acad. Sci. U. S. A* **97**, 3485-3490 (2000).
587. Fadok,V.A., Warner,M.L., Bratton,D.L. & Henson,P.M. CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha v beta 3). *J. Immunol.* **161**, 6250-6257 (1998).
588. Flora,P.K. & Gregory,C.D. Recognition of apoptotic cells by human macrophages: inhibition by a monocyte/macrophage-specific monoclonal antibody. *Eur. J. Immunol.* **24**, 2625-2632 (1994).
589. Devitt,A. *et al.* Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature* **392**, 505-509 (1998).
590. Cunningham,M.D., Bajorath,J., Somerville,J.E. & Darveau,R.P. Escherichia coli and Porphyromonas gingivalis lipopolysaccharide interactions with CD14: implications for myeloid and nonmyeloid cell activation. *Clin. Infect. Dis.* **28**, 497-504 (1999).

591. Yang,R.B., Mark,M.R., Gurney,A.L. & Godowski,P.J. Signaling events induced by lipopolysaccharide-activated toll-like receptor 2. *J. Immunol.* **163**, 639-643 (1999).
592. Dziarski,R., Wang,Q., Miyake,K., Kirschning,C.J. & Gupta,D. MD-2 enables Toll-like receptor 2 (TLR2)-mediated responses to lipopolysaccharide and enhances TLR2-mediated responses to Gram-positive and Gram-negative bacteria and their cell wall components. *J. Immunol.* **166**, 1938-1944 (2001).
593. Schroder,N.W. *et al.* Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J. Biol. Chem.* **278**, 15587-15594 (2003).
594. Kopydlowski,K.M. *et al.* Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. *J. Immunol.* **163**, 1537-1544 (1999).
595. Bausinger,H. *et al.* Endotoxin-free heat-shock protein 70 fails to induce APC activation. *Eur. J. Immunol.* **32**, 3708-3713 (2002).
596. Henderson,B., Poole,S. & Wilson,M. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol. Rev.* **60**, 316-341 (1996).
597. Lee,H.K., Lee,J. & Tobias,P.S. Two lipoproteins extracted from *Escherichia coli* K-12 LCD25 lipopolysaccharide are the major components responsible for Toll-like receptor 2-mediated signaling. *J. Immunol.* **168**, 4012-4017 (2002).
598. Brightbill,H.D. *et al.* Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science* **285**, 732-736 (1999).
599. Rawadi,G. & Roman-Roman,S. Mycoplasma membrane lipoproteins induced proinflammatory cytokines by a mechanism distinct from that of lipopolysaccharide. *Infect. Immun.* **64**, 637-643 (1996).
600. Sellati,T.J. *et al.* *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides activate monocytic cells via a CD14-dependent pathway distinct from that used by lipopolysaccharide. *J. Immunol.* **160**, 5455-5464 (1998).
601. Levin,J. & Bang,F.B. The role of endotoxin in the extracellular coagulation of *Limulus* blood. *Bull. Johns. Hopkins. Hosp.* **115**, 265-274 (1964).
602. Novitsky,T.J., Roslansky,P.F., Siber,G.R. & Warren,H.S. Turbidimetric method for quantifying serum inhibition of *Limulus* amoebocyte lysate. *J. Clin. Microbiol.* **21**, 211-216 (1985).



603. Brooks,R.A., Wimhurst,J.A. & Rushton,N. Endotoxin contamination of particles produces misleading inflammatory cytokine responses from macrophages in vitro. *J. Bone Joint Surg. Br.* **84**, 295-299 (2002).
604. Hurley,J.C. Endotoxemia: methods of detection and clinical correlates. *Clin. Microbiol. Rev.* **8**, 268-292 (1995).
605. Novitsky,T.J. Limitations of the Limulus amebocyte lysate test in demonstrating circulating lipopolysaccharides. *Ann. N. Y. Acad. Sci.* **851**, 416-421 (1998).
606. NEWTON,B.A. The properties and mode of action of the polymyxins. *Bacteriol. Rev.* **20**, 14-27 (1956).
607. Srimal,S., Surolia,N., Balasubramanian,S. & Surolia,A. Titration calorimetric studies to elucidate the specificity of the interactions of polymyxin B with lipopolysaccharides and lipid A. *Biochem. J.* **315 ( Pt 2)**, 679-686 (1996).
608. Morrison,D.C. & Jacobs,D.M. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry.* **13**, 813-818 (1976).
609. Gao,B. & Tsan,M.F. Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages. *J. Biol. Chem.* **278**, 174-179 (2003).
610. Gao,B. & Tsan,M.F. Recombinant human heat shock protein 60 does not induce the release of tumor necrosis factor alpha from murine macrophages. *J. Biol. Chem.* **278**, 22523-22529 (2003).
611. Issekutz,A.C. Removal of gram-negative endotoxin from solutions by affinity chromatography. *J. Immunol. Methods* **61**, 275-281 (1983).
612. Tsuzuki,H., Tani,T., Ueyama,H. & Kodama,M. Lipopolysaccharide: neutralization by polymyxin B shuts down the signaling pathway of nuclear factor kappaB in peripheral blood mononuclear cells, even during activation. *J. Surg. Res.* **100**, 127-134 (2001).
613. Cavaillon,J.M. & Haeffner-Cavaillon,N. Polymyxin-B inhibition of LPS-induced interleukin-1 secretion by human monocytes is dependent upon the LPS origin. *Mol. Immunol.* **23**, 965-969 (1986).
614. Talmadge,K.W. & Siebert,C.J. Efficient endotoxin removal with a new sanitizable affinity column: Affi-Prep Polymyxin. *J. Chromatogr.* **476**, 175-185 (1989).
615. Anspach,F.B. & Hilbeck,O. Removal of endotoxins by affinity sorbents. *J. Chromatogr. A* **711**, 81-92 (1995).

616. Duff,G.W. & Atkins,E. The inhibitory effect of polymyxin B on endotoxin-induced endogenous pyrogen production. *J. Immunol. Methods* **52**, 333-340 (1982).
617. Liu,S. *et al.* Removal of endotoxin from recombinant protein preparations. *Clin. Biochem.* **30**, 455-463 (1997).
618. Anspach,F.B. Endotoxin removal by affinity sorbents. *J. Biochem. Biophys. Methods* **49**, 665-681 (2001).
619. Karplus,T.E., Ulevitch,R.J. & Wilson,C.B. A new method for reduction of endotoxin contamination from protein solutions. *J. Immunol. Methods* **105**, 211-220 (1987).
620. Wicks,I.P. *et al.* Bacterial lipopolysaccharide copurifies with plasmid DNA: implications for animal models and human gene therapy. *Hum. Gene Ther.* **6**, 317-323 (1995).
621. Kol,A., Lichtman,A.H., Finberg,R.W., Libby,P. & Kurt-Jones,E.A. Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells. *J. Immunol.* **164**, 13-17 (2000).
622. Norgard,M.V. *et al.* Activation of human monocytic cells by *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides proceeds via a pathway distinct from that of lipopolysaccharide but involves the transcriptional activator NF-kappa B. *Infect. Immun.* **64**, 3845-3852 (1996).
623. Erroi,A. *et al.* Interleukin-1 and tumor necrosis factor production in acute non-lymphoid leukemia. *Eur. J. Haematol.* **42**, 16-23 (1989).
624. Fibbe,W.E. *et al.* Factors influencing release of granulocyte-macrophage colony-stimulating activity from human mononuclear phagocytes. *Eur. J. Haematol.* **41**, 352-358 (1988).
625. Biragyn,A. *et al.* Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science* **298**, 1025-1029 (2002).
626. Hirayama,C. & Sakata,M. Chromatographic removal of endotoxin from protein solutions by polymer particles. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **781**, 419-432 (2002).
627. Shen,W.F. *et al.* Lineage-restricted expression of homeobox-containing genes in human hematopoietic cell lines. *Proc. Natl. Acad. Sci. U. S. A* **86**, 8536-8540 (1989).
628. Lu,S.J., Li,F., Vida,L. & Honig,G.R. Comparative gene expression in hematopoietic progenitor cells derived from embryonic stem cells. *Exp. Hematol.* **30**, 58-66 (2002).

629. Locati, M. *et al.* Analysis of the gene expression profile activated by the CC chemokine ligand 5/RANTES and by lipopolysaccharide in human monocytes. *J. Immunol.* **168**, 3557-3562 (2002).
630. Jones, B.W. *et al.* Different Toll-like receptor agonists induce distinct macrophage responses. *J. Leukoc. Biol.* **69**, 1036-1044 (2001).
631. Ojaniemi, M. *et al.* Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. *Eur. J. Immunol.* **33**, 597-605 (2003).
632. Ardeschna, K.M., Pizzey, A.R., Devereux, S. & Khwaja, A. The PI3 kinase, p38 SAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. *Blood* **96**, 1039-1046 (2000).
633. Ravasi, T. *et al.* Generation of diversity in the innate immune system: macrophage heterogeneity arises from gene-autonomous transcriptional probability of individual inducible genes. *J. Immunol.* **168**, 44-50 (2002).
634. Tapping, R.I., Akashi, S., Miyake, K., Godowski, P.J. & Tobias, P.S. Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for Escherichia and Salmonella lipopolysaccharides. *J. Immunol.* **165**, 5780-5787 (2000).
635. Hirschfeld, M., Ma, Y., Weis, J.H., Vogel, S.N. & Weis, J.J. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J. Immunol.* **165**, 618-622 (2000).
636. Manthey, C.L. & Vogel, S.N. Interactions of lipopolysaccharide with macrophages. *Immunol. Ser.* **60**, 63-81 (1994).
637. Manthey, C.L. *et al.* Endotoxin-induced early gene expression in C3H/HeJ (Lpsd) macrophages. *J. Immunol.* **153**, 2653-2663 (1994).
638. Sato, S. *et al.* Synergy and cross-tolerance between toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. *J. Immunol.* **165**, 7096-7101 (2000).
639. Gao, J.J. *et al.* Cutting edge: bacterial DNA and LPS act in synergy in inducing nitric oxide production in RAW 264.7 macrophages. *J. Immunol.* **163**, 4095-4099 (1999).
640. Gao, J.J. *et al.* Bacterial LPS and CpG DNA differentially induce gene expression profiles in mouse macrophages. *J. Endotoxin. Res.* **9**, 237-243 (2003).
641. Fan, J., Frey, R.S. & Malik, A.B. TLR4 signaling induces TLR2 expression in endothelial cells via neutrophil NADPH oxidase. *J. Clin. Invest* **112**, 1234-1243 (2003).

642. Kreutz,M. *et al.* A comparative analysis of cytokine production and tolerance induction by bacterial lipopeptides, lipopolysaccharides and *Staphylococcus aureus* in human monocytes. *Immunology* **92**, 396-401 (1997).
643. Zhang,H., Peterson,J.W., Niesel,D.W. & Klimpel,G.R. Bacterial lipoprotein and lipopolysaccharide act synergistically to induce lethal shock and proinflammatory cytokine production. *J. Immunol.* **159**, 4868-4878 (1997).
644. Ozinsky,A., Smith,K.D., Hume,D. & Underhill,D.M. Co-operative induction of pro-inflammatory signaling by Toll-like receptors. *J. Endotoxin. Res.* **6**, 393-396 (2000).
645. Beutler,E., Gelbart,T. & West,C. Synergy between TLR2 and TLR4: a safety mechanism. *Blood Cells Mol. Dis.* **27**, 728-730 (2001).
646. Cowdery,J.S., Chace,J.H., Yi,A.K. & Krieg,A.M. Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. *J. Immunol.* **156**, 4570-4575 (1996).
647. Sparwasser,T. *et al.* Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor-alpha-mediated shock. *Eur. J. Immunol.* **27**, 1671-1679 (1997).
648. Pinhal-Enfield,G. *et al.* An angiogenic switch in macrophages involving synergy between Toll-like receptors 2, 4, 7, and 9 and adenosine A(2A) receptors. *Am. J. Pathol.* **163**, 711-721 (2003).
649. Rothstein,J.L. & Schreiber,H. Synergy between tumor necrosis factor and bacterial products causes hemorrhagic necrosis and lethal shock in normal mice. *Proc. Natl. Acad. Sci. U. S. A* **85**, 607-611 (1988).
650. King,R.W. Roughing up Smoothened: chemical modulators of Hedgehog signaling. *J. Biol.* **1**, 8 (2002).
651. Frank-Kamenetsky,M. *et al.* Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. *J. Biol.* **1**, 10 (2002).
652. Taipale,J., Cooper,M.K., Maiti,T. & Beachy,P.A. Patched acts catalytically to suppress the activity of Smoothened. *Nature* **418**, 892-897 (2002).
653. Thomas,S. *et al.* Vascular endothelial growth factor receptors in human mesangium in vitro and in glomerular disease. *J. Am. Soc. Nephrol.* **11**, 1236-1243 (2000).
654. Pepinsky,R.B. *et al.* Mapping sonic hedgehog-receptor interactions by steric interference. *J. Biol. Chem.* **275**, 10995-11001 (2000).

655. Ziegler-Heitbrock, H.W. *et al.* In vitro desensitization to lipopolysaccharide suppresses tumour necrosis factor, interleukin-1 and interleukin-6 gene expression in a similar fashion. *Immunology* **75**, 264-268 (1992).
656. Erroi, A. *et al.* Differential regulation of cytokine production in lipopolysaccharide tolerance in mice. *Infect. Immun.* **61**, 4356-4359 (1993).
657. Fahmi, H. & Chaby, R. Selective refractoriness of macrophages to endotoxin-induced production of tumor necrosis factor, elicited by an autocrine mechanism. *J. Leukoc. Biol.* **53**, 45-52 (1993).
658. Fahmi, H. & Chaby, R. Desensitization of macrophages to endotoxin effects is not correlated with a down-regulation of lipopolysaccharide-binding sites. *Cell Immunol.* **150**, 219-229 (1993).
659. Haas, J.G., Baeuerle, P.A., Riethmuller, G. & Ziegler-Heitbrock, H.W. Molecular mechanisms in down-regulation of tumor necrosis factor expression. *Proc. Natl. Acad. Sci. U. S. A* **87**, 9563-9567 (1990).
660. Gao, B. & Tsan, M.F. Induction of cytokines by heat shock proteins and endotoxin in murine macrophages. *Biochem. Biophys. Res. Commun.* **317**, 1149-1154 (2004).
661. Karahashi, H. & Amano, F. Endotoxin-tolerance to the cytotoxicity toward a macrophage-like cell line, J774.1, induced by lipopolysaccharide and cycloheximide: role of p38 MAPK in induction of the cytotoxicity. *Biol. Pharm. Bull.* **26**, 1249-1259 (2003).
662. Kaufmann, A., Gerns, D. & Sprenger, H. Differential desensitization of lipopolysaccharide-inducible chemokine gene expression in human monocytes and macrophages. *Eur. J. Immunol.* **30**, 1562-1567 (2000).
663. Gratchev, A., Schledzewski, K., Guillot, P. & Goerdts, S. Alternatively activated antigen-presenting cells: molecular repertoire, immune regulation, and healing. *Skin Pharmacol. Appl. Skin Physiol* **14**, 272-279 (2001).
664. Petsch, D. & Anspach, F.B. Endotoxin removal from protein solutions. *J. Biotechnol.* **76**, 97-119 (2000).
665. Wachtel, R.E. & Tsuji, K. Comparison of limulus amebocyte lysates and correlation with the United States Pharmacopeial pyrogen test. *Appl. Environ. Microbiol.* **33**, 1265-1269 (1977).
666. Ohno, N. & Morrison, D.C. Lipopolysaccharide interactions with lysozyme differentially affect lipopolysaccharide immunostimulatory activity. *Eur. J. Biochem.* **186**, 629-636 (1989).
667. Ellass-Rochard, E. *et al.* Lactoferrin-lipopolysaccharide interaction: involvement of the 28-34 loop region of human lactoferrin in the high-

- affinity binding to Escherichia coli 055B5 lipopolysaccharide. *Biochem. J.* **312** ( Pt 3), 839-845 (1995).
668. Berger,D. & Beger,H.G. Comparison of the endotoxin-binding capacity of human transferrin and a human applicable immunoglobulin preparation. *Arzneimittelforschung.* **38**, 817-820 (1988).
  669. Kaca,W., Roth,R.I. & Levin,J. Hemoglobin, a newly recognized lipopolysaccharide (LPS)-binding protein that enhances LPS biological activity. *J. Biol. Chem.* **269**, 25078-25084 (1994).
  670. Takeuchi,Y. & Nikaido,H. Physical interaction between lipid A and phospholipids: a study with spin-labeled phospholipids. *Rev. Infect. Dis.* **6**, 488-492 (1984).
  671. Shands,J.W., Jr. Affinity of endotoxin for membranes. *J. Infect. Dis.* **128**, Suppl-201 (1973).
  672. Harris,H.W., Eichbaum,E.B., Kane,J.P. & Rapp,J.H. Detection of endotoxin in triglyceride-rich lipoproteins in vitro. *J. Lab Clin. Med.* **118**, 186-193 (1991).
  673. Levin,J., Tomasulo,P.A. & Oser,R.S. Detection of endotoxin in human blood and demonstration of an inhibitor. *J. Lab Clin. Med.* **75**, 903-911 (1970).
  674. Hochstein,H.D., Seligmann,E.B., Jr., Marquina,R.E. & Rivera,E. Limulus amebocyte lysate testing of normal serum albumin (human) in the United States since 1975. *Dev. Biol. Stand.* **44**, 35-42 (1979).
  675. Petsch,D., Deckwer,W.D. & Anspach,F.B. Proteinase K digestion of proteins improves detection of bacterial endotoxins by the Limulus amebocyte lysate assay: application for endotoxin removal from cationic proteins. *Anal. Biochem.* **259**, 42-47 (1998).
  676. Wallin,R.P. *et al.* Heat-shock proteins as activators of the innate immune system. *Trends Immunol.* **23**, 130-135 (2002).
  677. Kirikae,T. *et al.* Lipopolysaccharides (LPS) of oral black-pigmented bacteria induce tumor necrosis factor production by LPS-refractory C3H/HeJ macrophages in a way different from that of Salmonella LPS. *Infect. Immun.* **67**, 1736-1742 (1999).
  678. Vaara,M. *et al.* Characterization of the lipopolysaccharide from the polymyxin-resistant pmrA mutants of Salmonella typhimurium. *FEBS Lett.* **129**, 145-149 (1981).
  679. Hogasen,A.K. & Abrahamsen,T.G. Polymyxin B stimulates production of complement components and cytokines in human monocytes. *Antimicrob. Agents Chemother.* **39**, 529-532 (1995).

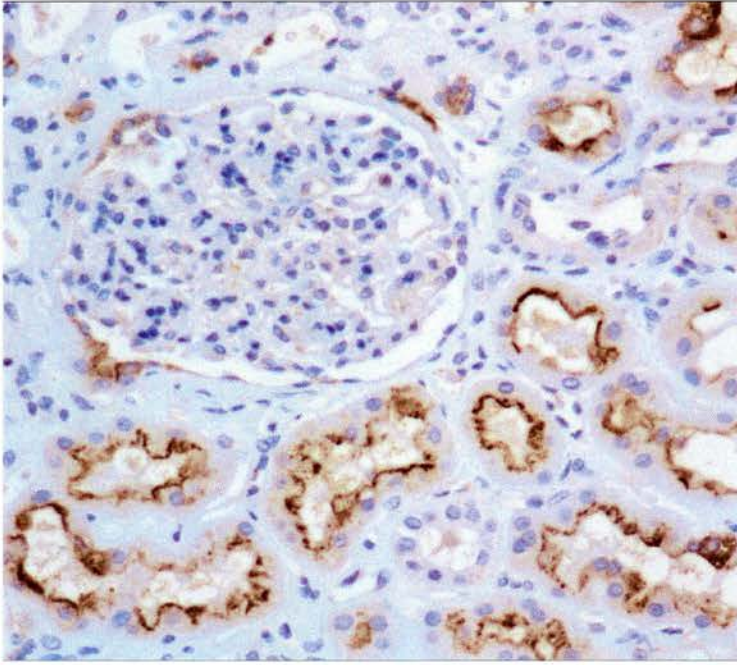


680. Jaber,B.L., Sundaram,S., Cendoroglo,N.M., King,A.J. & Pereira,B.J. Polymyxin-B stimulates tumor necrosis factor-alpha production by human peripheral blood mononuclear cells. *Int. J. Artif. Organs* **21**, 269-273 (1998).
681. Morrison,D.C. & Curry,B.J. The use of polymyxin B and C3H/HeJ mouse spleen cells as criteria for endotoxin contamination. *J. Immunol. Methods* **27**, 83-92 (1979).
682. Goodrich,L.V. & Scott,M.P. Hedgehog and patched in neural development and disease. *Neuron* **21**, 1243-1257 (1998).
683. Manyonda,I.T., Soltys,A.J. & Hay,F.C. A critical evaluation of the magnetic cell sorter and its use in the positive and negative selection of CD45RO+ cells. *J. Immunol. Methods* **149**, 1-10 (1992).
684. Jacobs,N., Moutschen,M.P., Boniver,J., Greimers,R. & Schaaf-Lafontaine,N. Efficient immunoselection of cytolytic effectors with a magnetic cell sorter. *Res. Immunol.* **144**, 141-150 (1993).
685. Stanciu,L.A., Shute,J., Holgate,S.T. & Djukanovic,R. Production of IL-8 and IL-4 by positively and negatively selected CD4+ and CD8+ human T cells following a four-step cell separation method including magnetic cell sorting (MACS). *J. Immunol. Methods* **189**, 107-115 (1996).
686. Black,A.P., Bhayani,H., Ryder,C.A., Gardner-Medwin,J.M. & Southwood,T.R. T-cell activation without proliferation in juvenile idiopathic arthritis. *Arthritis Res.* **4**, 177-183 (2002).
687. Caruso,A. *et al.* Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. *Cytometry* **27**, 71-76 (1997).
688. Johannisson,A., Thuvander,A. & Gadhasson,I.L. Activation markers and cell proliferation as indicators of toxicity: a flow cytometric approach. *Cell Biol. Toxicol.* **11**, 355-366 (1995).
689. Sloan-Lancaster,J. & Allen,P.M. Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. *Annu. Rev. Immunol.* **14**, 1-27 (1996).
690. Horejsi,V. *et al.* GPI-microdomains: a role in signalling via immunoreceptors. *Immunol. Today* **20**, 356-361 (1999).
691. Fragoso,R. *et al.* Lipid raft distribution of CD4 depends on its palmitoylation and association with Lck, and evidence for CD4-induced lipid raft aggregation as an additional mechanism to enhance CD3 signaling. *J. Immunol.* **170**, 913-921 (2003).

692. Arcaro,A. *et al.* Essential role of CD8 palmitoylation in CD8 coreceptor function. *J. Immunol.* **165**, 2068-2076 (2000).
693. Gelman,A.E., Zhang,J., Choi,Y. & Turka,L.A. Toll-like receptor ligands directly promote activated CD4+ T cell survival. *J. Immunol.* **172**, 6065-6073 (2004).
694. Musikacharoen,T., Matsuguchi,T., Kikuchi,T. & Yoshikai,Y. NF-kappa B and STAT5 play important roles in the regulation of mouse Toll-like receptor 2 gene expression. *J. Immunol.* **166**, 4516-4524 (2001).
695. Matsuguchi,T., Takagi,K., Musikacharoen,T. & Yoshikai,Y. Gene expressions of lipopolysaccharide receptors, toll-like receptors 2 and 4, are differently regulated in mouse T lymphocytes. *Blood* **95**, 1378-1385 (2000).
696. Mokuno,Y. *et al.* Expression of toll-like receptor 2 on gamma delta T cells bearing invariant V gamma 6/V delta 1 induced by Escherichia coli infection in mice. *J. Immunol.* **165**, 931-940 (2000).
697. Caramalho,I. *et al.* Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J. Exp. Med.* **197**, 403-411 (2003).
698. Liew,F.Y., Komai-Koma,M. & Xu,D. A toll for T cell costimulation. *Ann. Rheum. Dis.* **63 Suppl 2**, ii76-ii78 (2004).
699. Henderson,B., Poole,S. & Wilson,M. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol. Rev.* **60**, 316-341 (1996).
700. Vogel,S.N., Hilfiker,M.L. & Caulfield,M.J. Endotoxin-induced T lymphocyte proliferation. *J. Immunol.* **130**, 1774-1779 (1983).
701. Watanabe,T. *et al.* Lipid A directly inhibits IL-4 production by murine Th2 cells but does not inhibit IFN-gamma production by Th1 cells. *Eur. J. Immunol.* **29**, 413-418 (1999).
702. Ismaili,J. *et al.* Monophosphoryl lipid A activates both human dendritic cells and T cells. *J. Immunol.* **168**, 926-932 (2002).
703. Takada,H., Hiromatsu,K., Matsuzaki,G., Muramori,K. & Nomoto,K. Peritoneal gamma delta T cells induced by Escherichia coli infection in mice. Correlation between Thy-1 phenotype and host minor lymphocyte-stimulating phenotype. *J. Immunol.* **151**, 2062-2069 (1993).
704. Takada,H., Matsuzaki,G., Yoshida,H., Sumichika,H. & Nomoto,K. Functional difference between Thy-1-positive and Thy-1-negative gamma delta T cells induced by Escherichia coli infection in mice. *Immunology* **88**, 261-268 (1996).

705. Messina,J.P., Gilkeson,G.S. & Pisetsky,D.S. Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA. *J. Immunol.* **147**, 1759-1764 (1991).
706. Charron,F., Stein,E., Jeong,J., McMahon,A.P. & Tessier-Lavigne,M. The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. *Cell* **113**, 11-23 (2003).
707. Marti,E. & Bovolenta,P. Sonic hedgehog in CNS development: one signal, multiple outputs. *Trends Neurosci.* **25**, 89-96 (2002).

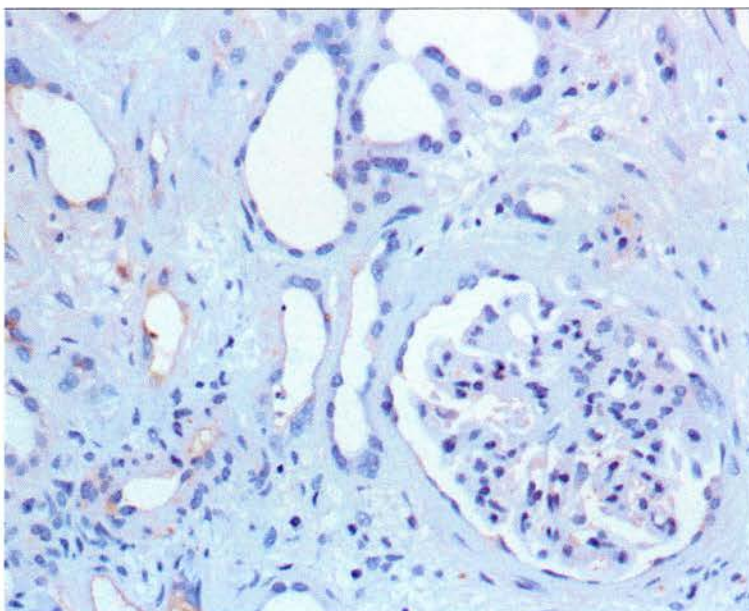
## **Appendices**



## **APPENDIX 1**

### **Megalin is expressed on the apical surface of proximal convoluted tubular cells**

Sections of normal human kidney were investigated for the expression of megalin. Immunohistochemistry was performed as outlined in Chapter 2 using a goat polyclonal antibody to megalin (Santa Cruz) at a dilution of 1:40. This confirmed the expression of megalin on the apical surface of proximal convoluted tubular cells (Mag x 400), a finding in keeping with other reports in the literature. Results represent experiments performed on renal tissue from 2 donors.

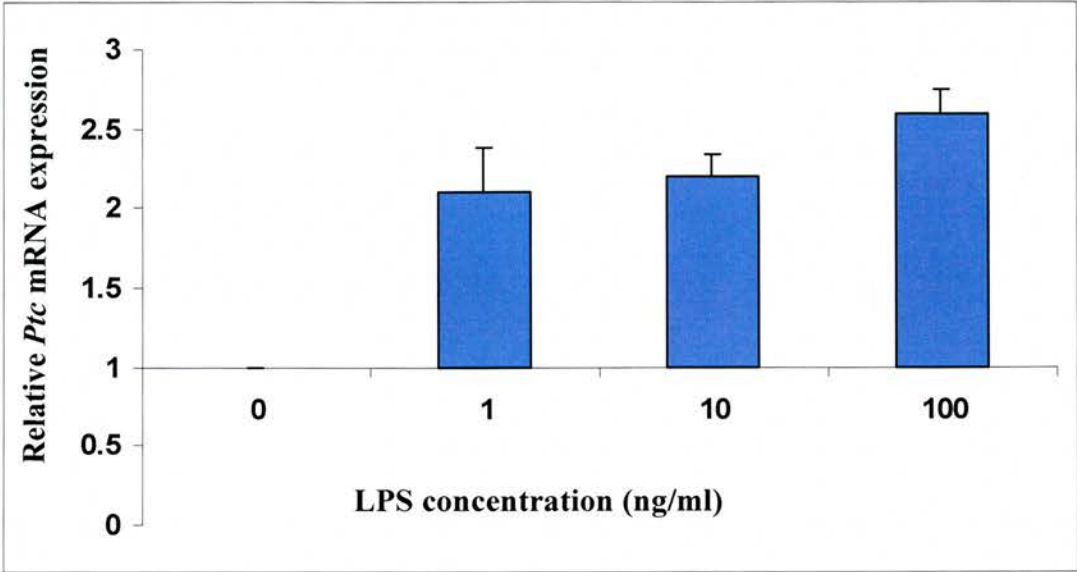


## **APPENDIX 2**

### **Megalin fails to be expressed in CAN**

Sections of CAN were investigated for the expression of megalin. Immunohistochemistry was performed as outlined in Chapter 2 using a goat polyclonal antibody to megalin (Santa Cruz) at a dilution of 1:40. This failed to demonstrate the presence of megalin in samples of CAN (Mag x 400). Results represent experiments performed on renal tissue from 5 donors.



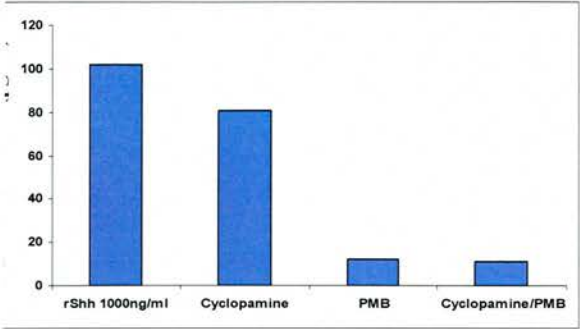


**APPENDIX 3**

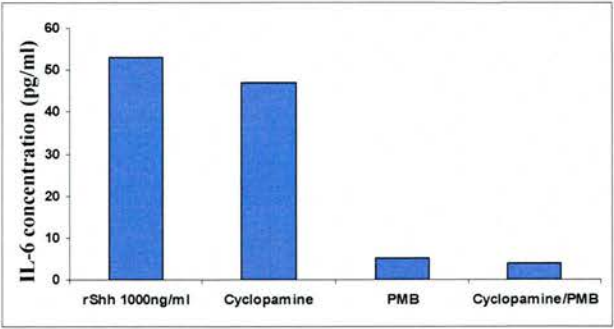
***Ptc* expression is upregulated in macrophages in response to LPS**

Real-time PCR was performed to determine the expression of *Ptc* mRNA in macrophages in response to 1-100ng/ml *E coli* 055:B5 LPS following an 18 hour incubation. Values are expressed as a ratio of the concentration of *Ptc* mRNA relative to untreated macrophages grown in medium alone. The untreated (0ng/ml LPS) macrophages are given a value of 1. *Ptc* mRNA expression is upregulated in response to treatment with LPS. Results represent experiments performed on 2 donors.

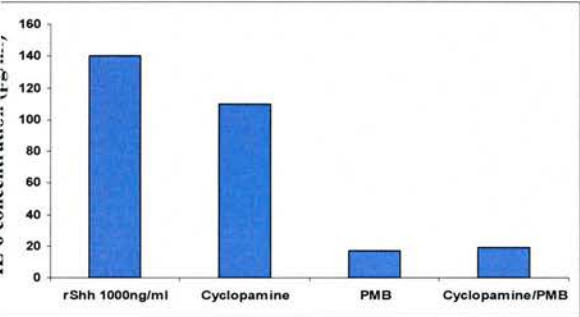
**Donor 1 – IMDM-FCS**



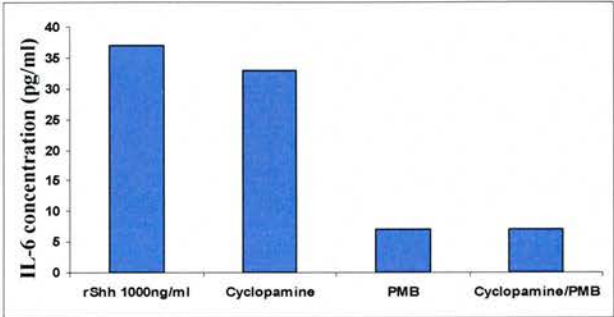
**Donor 1 – X-vivo 10**



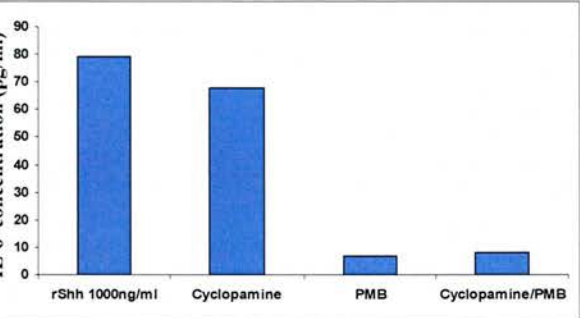
**Donor 2 – IMDM-FCS**



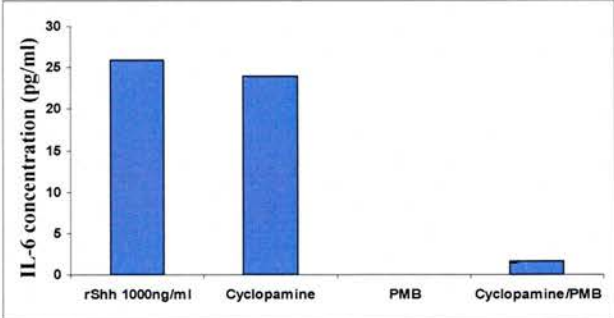
**Donor 2 – X-vivo 10**



**Donor 3 – IMDM-FCS**



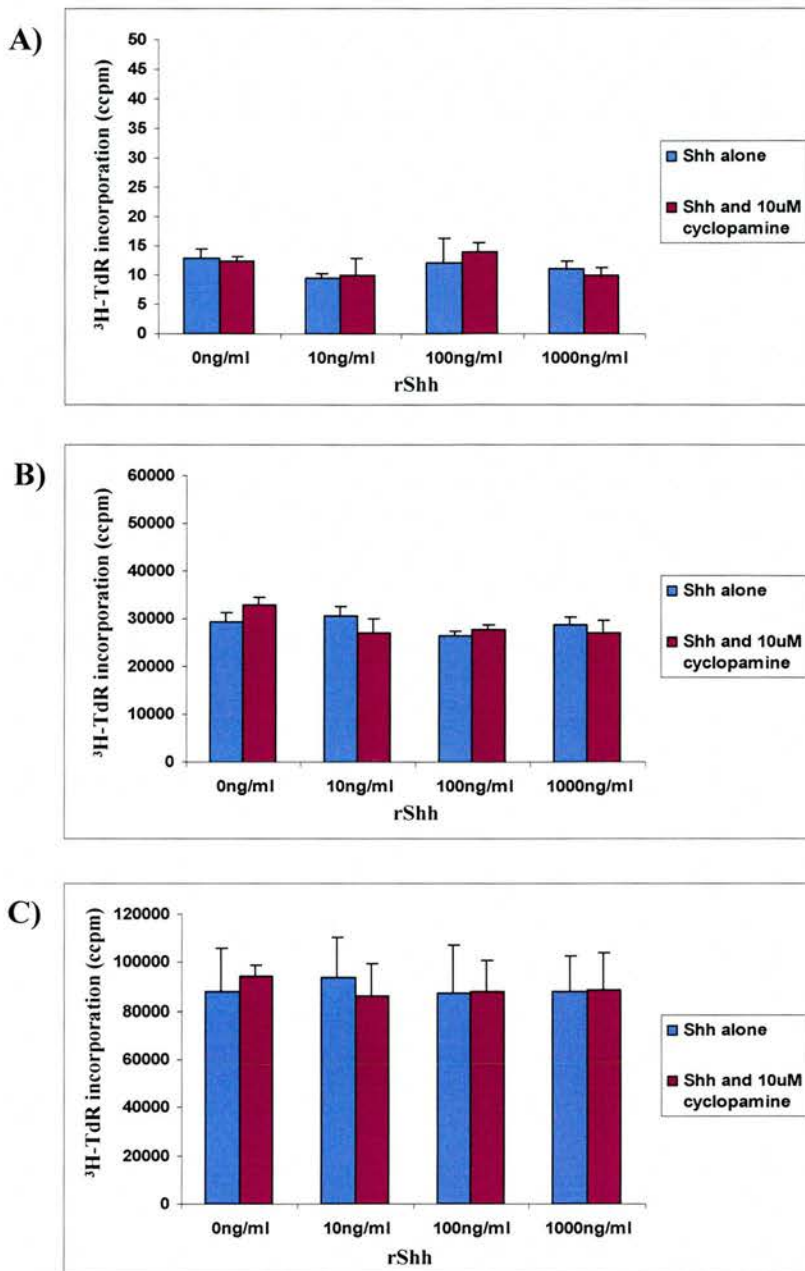
**Donor 3 – X-vivo 10**



**APPENDIX 4**

**The inhibitory effects of cyclopamine and polymixin B are not additive**

Macrophages were pre-incubated with either 10μM cyclopamine, 10μg/ml polymixin B or both 10μM cyclopamine and 10μg/ml polymixin B for 45 minutes prior to the addition of rShh. rShh was added to give a final concentration of 1000ng/ml and the cells were incubated in these conditions for 24 hours. The supernatants were harvested after 24 hours and CBAs employed to quantify cytokine and chemokine production. The graphs above illustrate IL-6 production under the conditions noted above for 3 donors in both serum-containing (IMDM-FCS) and serum-free (X-vivo 10) conditions.



## APPENDIX 5

### Cyclopamine fails to modulate the response of cell-sorted CD4+ T cells to Shh

Preliminary experiments were performed in two human T cell donors to determine the response to CD4+ T cells to rShh when pre-incubated with the specific inhibitor of the Shh signalling pathway, cyclopamine.  $2 \times 10^5$  FACS sorted CD4+ T cells per well were pre-incubated for 45 minutes at 37°C with 10µM cyclopamine. rShh was then added to the wells to give a final concentration of 10-1000ng/ml rShh. Each experiment was repeated in quadruplicate. As previously, the cells were incubated for a period of 72 hours. At the end of this incubation period, 20µl of culture medium was removed from each well for later cytokine analysis. The cells were pulsed with  $^3\text{H}$ -TdR (1µCi/well) and incubated for a further 18 hours.  $^3\text{H}$ -TdR incorporation was evaluated to assess proliferation of the T cells. Results represent the proliferation experiments performed on 2 donors. As shown, resting (A), suboptimally activated (B) and optimally activated (C) CD4+ T cell proliferation were unaffected by cyclopamine. Similarly cytokine effector function was not modulated by cyclopamine administration in either of the donors (data not shown).

## APPENDIX 6

### Publications arising from work presented in this thesis:

- Wakelin SJ, Marson L, Howie SEM, Garden OJ, Lamb JR, Forsythe JRL. The Role of Vascular Endothelial Growth Factor in the Kidney in Health and Disease. *Nephron Physiol* 2004; **98**:73-79.
- Fitch PM, Wakelin SJ, Lowrey JA, Wallace WAH, Howie SEM. Chapter 18 on 'Shh Expression in Pulmonary Injury and Disease' in Hedgehog-Gli Signalling and Human Disease.
- Stewart GA, Lowrey JA, Wakelin SJ, Fitch PM, Lindey S, Dallman MJ, Lamb JR, Howie SEM. Sonic hedgehog signalling modulates activation of and cytokine production by human peripheral CD4<sup>+</sup> T cells. *J Immunology* 2002; **169**:5451-7.

# The Role of Vascular Endothelial Growth Factor in the Kidney in Health and Disease

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## Key Words

vascular endothelial growth factor · Renal development · Glomerular disease

## Abstract

Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen, angiogenic factor and enhancer of vascular permeability. Expressed in the epithelial cells of the developing glomerulus and tubular epithelium, VEGF plays an important role in the development and maintenance of the early vasculature of the kidney. Here, we review the available literature regarding the expression and function of VEGF both in the developing and healthy adult kidney. Furthermore, we highlight how VEGF expression is altered in the diseased kidney and how this modulated expression may impact on and reflect underlying functional changes occurring during the disease process. As discussed, many controversial issues remain, particularly concerning the role of VEGF in the diseased kidney. That VEGF has been proposed as a potential future therapeutic target for the management of some renal diseases requires first that the precise role of VEGF in the normal kidney and various renal pathologies be further and more clearly defined.

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## Introduction

In 1983, Senger et al. first described a protein secreted from tumours that increased the permeability of vessels [1]. This factor, originally named Vascular Permeability Factor, was later renamed Vascular Endothelial Growth Factor (VEGF) following the observation that, in addition to its effect on vascular permeability, it acted as a powerful angiogenic factor and a specific endothelial cell mitogen [2]. Given its action as an endothelial cell mitogen and angiogenic factor, much attention has focused on possible roles for VEGF in a number of physiological and pathophysiological processes including organogenesis, wound healing and tumour growth. However, these functions also suggest that VEGF may play an important role at sites where maintenance of a capillary bed may be critical for function. One of the best examples of such a capillary bed is in the kidney where an intact capillary network is integral to the filtration function of the nephron. The aim of this paper is to review the current understanding of VEGF and its receptors with particular reference to potential roles for the growth factor in the kidney in health and disease.



## VEGF and Its Receptors

VEGF, also referred to as VEGF-A, was the first member of the VEGF family to be identified [1]. Other family members now include VEGF B-E and placental growth factor. The gene for VEGF is located on chromosome 6p21.3. Containing 8 exons, the coding region spans 1.4 kb and alternative splicing of this gene gives rise to several isoforms encoding polypeptides of 121, 145, 148, 165, 183, 189 and 206 amino acids [3, 4]. A further isoform, 165b, is unusual in being proposed as an endogenous inhibitory isoform of VEGF [5]. The different isoforms vary in their ability to bind heparin, a property dependent on exon 6 and 7 expression. For example, VEGF189 and 206 contain both exons, bind heparin strongly and, once produced, they remain attached to extracellular matrix heparan sulphates. In contrast, VEGF165 and VEGF 145 have exon 7 and exon 6, respectively, and VEGF 121 contains neither exon. These are the more freely diffusible forms of VEGF.

VEGF is expressed by and secreted from a wide variety of cells and several variants may be expressed by one cell type simultaneously [6]. The repertoire of VEGF-producing cells includes macrophages, neutrophils, fibroblasts, platelets, eosinophils, mast cells, vascular smooth muscle cells, hepatocytes, epithelial cells, several tumours and all lines and endothelial cells in vitro. Most VEGF-producing cells preferentially express VEGF variants 121, 145 and 189, the other isoforms being less ubiquitously expressed.

VEGF, once secreted, may bind to several functionally distinct receptors, including VEGFR1 (Flt1), VEGFR2 (Flk1), VEGFR3 and neuropilin-1. Binding to these receptors mediates a variety of effects. VEGF is a potent endothelial cell mitogen, and mediator of angiogenesis [reviewed in 7]. In the embryo, it appears to be essential for normal vascular development, and mice, homo- or heterozygous for transgenic mutations that inactivate VEGF, die from vascular abnormalities in utero [8]. Conversely, embryos subjected to excessively high levels of VEGF exhibit hyperproliferation of vessels resulting in the formation of dysfunctional vessels and impaired organogenesis [9].

VEGF has profound effects on permeability [10], an effect possibly mediated via an increase in fenestration formation [11], the induction of transcellular gaps [12] the formation of caveolae [13] and the formation of interendothelial cell gaps [14]. Furthermore, VEGF acts as an endothelial cell survival factor [15], effects vasodilatation, probably via nitric oxide (NO) synthesis [16], and acts as

chemoattractant for monocytes [17]. It may also affect the maturation of dendritic cells [18] and the polarisation of T cells [19] and, thus, play a role in immune responses.

A multitude of factors upregulate VEGF at the mRNA level or affect VEGF release from cells. One of the strongest signals for VEGF expression is hypoxia, but other factors also upregulate VEGF. These include growth factors, cytokines, interleukins, prostaglandins, angiotensin II, adenosine, NO and hypo- and hyperglycaemia.

## Expression of VEGF in the Kidney

VEGF expression has been reported in several cell types within the human and rodent kidney. Most consistently, VEGF has been observed in the podocytes [20–22]. Outwith the glomerulus, VEGF expression has been detected in the renal tubular cells, distal more commonly than proximal [20, 23], and the collecting ducts [21]. In the developing kidney, VEGF is expressed in the podocyte, tubular epithelium, collecting ducts and mesenchymal cells [21].

Other areas of expression have been more controversial. For example, although several groups have reported the expression of VEGF by glomerular endothelial cells in vitro [24], many in situ localisation studies have failed to identify endothelial expression of VEGF [20, 25]. Furthermore, mesangial expression has been reported in vitro [26] and in models of disease [27, 28] but not in normal kidney [20, 25].

## Expression of Receptors for VEGF in the Kidney

VEGFR1 and 2 expression have been demonstrated, using  $^{125}\text{I}$ -VEGF<sub>165</sub> binding experiments and immunolocalisation in glomerular endothelial cells, peritubular capillaries and in pre- and post-glomerular vessels in the human and rodent kidney [21–23]. Neuropilin-1 is also expressed in the visceral epithelial cells of the glomerulus [29], mesangial cells [30] and in the afferent arterioles and glomerular capillaries [29].

## Functions of VEGF in the Normal Kidney

The enhanced expression of VEGF in the embryonic kidney suggests that it may have a specific role in renal development and, given its persistent presence in the



ult, it is tempting to speculate that it may continue to be involved in the mature kidney.

### *Development*

The definitive kidney or metanephros appears around E11 in the mouse and renal development continues into the second post-natal week. The excretory units develop from the metanephric mesoderm, and the collecting ducts from the ureteric bud, an outgrowth from the mesonephric duct. The ureteric bud invades the metanephric tissue, and islands of the latter cap the distal ends of the branching ureteric bud. The cells of the metanephric tissue cap are induced to become aggregates of epithelial cells. These abundantly express VEGF [21] and form small vesicles which subsequently give rise to comma- and S-shaped bodies. Small clefts develop, representing entry points for the developing glomerular vasculature probably occurring through a combination of angiogenesis and vasculogenesis. The former involves the sprouting of new vessels from pre-existing vessels and the latter involves the in situ differentiation of endothelial cell precursors (angioblasts) which then form vessels through coalescence.

It was previously thought that the metanephric vasculature was derived from angiogenesis. However, experiments involving renal grafts implanted into the anterior chamber of the eye suggested that VEGFR1- and VEGFR2-expressing angioblasts are primarily responsible for microvessel development in the kidney [31] suggesting a role for VEGF in the development process. Other work demonstrated that VEGF could convert a VEGFR2(-) precursor cell to a VEGFR2(+) haemangioblast [32] in vitro and, in metanephric organ cultures, high levels of VEGF were present in the medium at active stages of glomerulogenesis [33]. Furthermore, the differentiation of angioblasts into endothelial cells could be blocked by the administration of antibodies to VEGF [34]. Podocyte selective deletion of VEGF results in marked endotheliosis and progression to the nephrotic syndrome in heterozygotes. Homozygotes die soon after birth with renal failure and abnormal glomeruli lacking mature endothelial cells [35]. Overexpression of VEGF in the same system resulted in end-stage renal failure due to a collapsing glomerulopathy.

VEGF may also play a role later in the neonatal period with continued development of the kidney. Very high levels of VEGFR2 expression were demonstrated in the developing glomerular endothelium [34, 36], and blocking VEGF with antibodies in newborn mice resulted in abnormal glomerulogenesis, decreased glomerular formation and a significant reduction in glomerular vascularity

[33]. Furthermore, in antibody-treated mice, the capillaries failed to create useful glomerular vascular networks, suggesting that an intact VEGF gradient may be necessary to sustain rapid glomerular growth and development within a limited time frame [33].

### *Adult*

In the adult, renal VEGF expression persists albeit at a much lower level than that seen in the developing kidney [21, 36]. Given the pattern of VEGF and VEGFR expression in the adult kidney, it is possible that VEGF has a specific functional role, particularly in the glomerulus.

The glomerular capillaries are structurally different from other peripheral capillaries. The luminal layer is covered with fenestrated endothelium, which is unusual in not possessing bridging diaphragms, a finding in contrast to most other types of endothelial cell fenestrations. The endothelial cells are attached to the glomerular basement membrane consisting of extracellular matrix proteins. Outside the capillaries lie the podocytes, highly specialised cells with processes extending from the cell body providing support for the glomerular capillary network and synthesising the glomerular basement membrane. The glomerular capillary network is also supported by the mesangium (mesangial cells and extracellular matrix).

Several possible roles for VEGF signalling in the glomerulus have been proposed. That its role is outwith one of angiogenesis is suggested by the predominance of VEGFR1, rather than VEGFR2, in the glomerulus [22]. It may be that VEGF plays a role in regulating glomerular circulation and the maintenance of normal renal function [37]. The VEGF-producing podocytes sit in very close proximity to the glomerular endothelium expressing the VEGF receptors [21] with only the glomerular basement membrane interposed between them. For VEGF to bind to its receptors on the endothelium it must move upstream against the established ultrafiltration gradient tending to move water and solutes from the capillaries into the Bowman's space. It is therefore not clear whether or not VEGF signalling can occur. With data showing that cultured glomerular epithelial cells produce heparin-binding variants of VEGF, Chen et al. [13] proposed that VEGF produced by the glomerular epithelial cells may be deposited into the basement membrane, thus acting as a site for VEGF accumulation and storage. Alternatively, VEGF may act in an autocrine manner on podocytes, as evidenced by recent studies suggesting altered calcium handling and reduced cell death resulted when podocytes were cultured in the presence of VEGF [38].

Given that VEGF was originally described as a permeability factor and that selective filtration or permselectivity is a property integral to the glomerulus, it has been postulated that VEGF may have a role in this filtration process [20, 21, 25]. VEGF is known to induce fenestrations in endothelial cells in vitro [11]. It has also been reported to induce transcellular gaps [12], caveolae [13] and interendothelial gaps [14], and it has been suggested that VEGF may be involved in the induction and maintenance of the fenestrations in the capillaries of the kidney [22]. In support of this, VEGF inhibition was shown to reduce the number of fenestrations in glomerular endothelial cells [39]. In contrast, in normal kidney, and using VEGF antagonists, others have failed to demonstrate an effect of VEGF<sub>165</sub> on the constitutive fenestration of the glomerulus [40], suggesting that it is not required to maintain the fenestrated endothelial phenotype.

Irrespective of any possible role for VEGF in mediating endothelial cell fenestration, this may have limited importance in mediating glomerular permselectivity. Endothelial fenestrations may only be partly involved, if at all, in mediating filtration. Indeed, the glomerular BM, the negatively charged surface of the endothelial and epithelial cells and the podocytes with their interconnecting slit diaphragms may prove more of a barrier than the endothelial cells. However, VEGF may play some role in determining the composition of the glomerular basement membrane as evidenced by its ability to induce extracellular proteolysis, a function integral to its role as an angiogenic factor [41].

It is not entirely clear whether VEGF can truly affect the normal filtration of the glomerulus. VEGF is a potent vasodilator through its action on NO synthesis and thus has the potential to have profound effects on end-organ blood flow. In the kidney, such an effect will inevitably lead to enhanced macromolecular extravasation irrespective of any effects on permeability per se. Indeed, inhibitors of NO synthase are reported to increase renal vascular resistance and decrease the glomerular ultrafiltration coefficient [42]. Nevertheless, there is some evidence to suggest that VEGF may play a role in maintaining the normal filtration barrier of the kidney [43–46]. In normal healthy mice and pregnant rats, it has been demonstrated that VEGF blockade induces proteinuria in association with glomerular structural changes [43, 45]. In a clinical study, the monoclonal VEGF antibody bevacizumab was shown to induce proteinuria in some patients [44] and, more recently, in a large study of the general population in the Netherlands, elevated plasma VEGF levels were

associated with microalbuminuria [46]. Conversely, however, in other studies, VEGF antagonism using aptamers failed to induce glomerular pathology or proteinuria [40], and in isolated perfused rat kidneys, the glomerular permeability for albumin was not apparently affected by VEGF [47]. Thus, it would seem there is some headway to be made to determine what role, if any, VEGF plays in maintaining the normal filtration barrier of the glomerulus.

VEGF may play a role in aspects of nephron function outwith possible glomerular functions. For example, there are reports of VEGF expression in the distal and proximal convoluted tubules [6, 20, 23] and in the collecting ducts [21]. Thus, it is possible that VEGF may modulate the selective permeability in the tubules and collecting duct system or maintain the endothelium of the peritubular capillaries. Whether this is in fact the case has yet to be determined.

## VEGF and the Diseased Kidney

A multitude of pathologies can damage the kidney. Some, such as minimal change nephrotic syndrome, may be benign and reversible whilst others, including forms of glomerulonephritis and diabetic nephropathy can cause irreparable damage ultimately leading to end stage renal failure.

### *Acute Renal Damage*

Although conflicting results exist, there is a body of evidence to suggest that VEGF may be upregulated in the early stages of a number of pathologies, including several models of acute glomerulonephritis [23, 48] and early diabetic nephropathy [49, 50].

It is tempting to speculate that upregulated VEGF may play a reparatory role in the early stages of renal disease, perhaps in an attempt to recapitulate processes occurring in the embryo. No more so has this proved such an attractive possibility as for the glomerulus. Glomerular damage is common to many forms of renal pathology and, irrespective of the underlying injury, capillary destruction is almost invariable. In many experimental models of glomerular injury, simple damage to the glomerulus is usually followed by a period of repair and recovery of glomerular structure and function [51, 52]. Exogenous VEGF appears to enhance this process [53–55] whilst VEGF abrogation inhibits glomerular repair [28, 40].

The reparatory role of VEGF in these early disease states may not be limited to its mitogenic effect on glo-

glomerular endothelial cells. VEGF has been shown to induce tubular proliferation in metanephric organ culture experiments [34], and work in the remnant kidney model suggests that VEGF may have a tubuloprotective effect [53].

From the above observations, it seems possible that VEGF may be responsible for initiating an element of repair in the acutely and minimally damaged kidney. Whether these models of glomerular disease reflect the true situation in vivo however, is not clear. Indeed, most glomerular diseases are insidious rather than acute in nature and thus extrapolating the findings from these acute glomerular insult models to clinical disease must proceed with caution.

#### *Progressive Renal Disease*

Although not fully characterised, progressive renal disease is likely to reflect a multifactorial process with a common endpoint. Persistent injury, subsequent reduction in functioning nephron mass and the inability to sustain adequate glomerular filtration rate and the progressive loss of glomerular and interstitial microvasculature are all believed to be critical factors in the development of chronic renal disease.

Whilst VEGF is apparently upregulated in acute insults to the kidney, most studies would suggest that reduced VEGF expression is associated with progressive and advanced renal disease [40, 54, 56–60]. Human immunolocalisation studies have shown reduced or absent VEGF expression in a variety of advanced renal diseases [23, 60], and work in rodent models, including models of thrombotic microangiopathy (anti-glomerular endothelial cell antibody model) [55], tubulointerstitial injury (unilateral ureteral obstruction model) [57] and chronic renal failure (remnant kidney model) [53], has corroborated these findings.

Several groups have made the observation that the downregulation of tissue VEGF expression coincides with the infiltration of inflammatory cells into the kidney [53, 56, 57, 61]. Indeed Kang et al. [53] found that several macrophage-associated cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) inhibited VEGF mRNA expression and protein secretion by cultured tubular cells under both normoxic and hypoxic conditions. If macrophage products reduce the VEGF expression of tubular cells in vivo, it may be postulated that this will have an impact on the development of interstitial fibrosis, particularly if tubular VEGF is cytoprotective.

As alluded to above, it is not altogether clear what mediates disease progression in the kidney. Although un-

likely to apply universally to all forms of renal pathology, one attractive and longstanding hypothesis is that, in response to progressive disease, the kidney attempts to compensate for the resulting inadequate glomerular filtration through a combination of glomerular hypertension and hypertrophy in the remaining nephrons [62]. Glomerular hypertension may then cause further damage by injuring the glomerular endothelium and mesangium, thus precipitating proteinuria.

There are interesting reports suggesting a possible role for VEGF in these elements of disease progression. For example, in mechanical stretch models of intraglomerular hypertension, early mesangial cell VEGF upregulation has been observed [27], providing a potential molecular mechanism by which a haemodynamic insult might translate into increased filtration of macromolecules resulting in proteinuria. Although this would suggest a possible link between glomerular hypertension with VEGF, is there any good evidence linking VEGF with proteinuria in disease? Proteinuria is an adverse prognostic feature in advancing renal disease and there is some evidence to suggest that VEGF may induce proteinuria in non-diseased kidneys [43–45]. However, there is marked variation between studies reporting on the relationship between VEGF and proteinuria in diseased kidneys. Indeed, both upregulated [48, 63, 64] and downregulated [65] VEGF have been associated with proteinuria in various diseases. In disease models, VEGF administration is mostly unsuccessful in inducing proteinuria [66, 67] and, indeed, several studies have shown no relationship between proteinuria and VEGF expression [65–69].

#### **Conclusions**

From the evidence presented in this review, it appears that VEGF may play a number of roles. Whilst a role for VEGF in the embryonic kidney is being increasingly well defined, the role of VEGF in the normal adult kidney is less clear. Studies of acute renal damage using animal models would suggest that simple reversible damage to the finely balanced capillary network can be managed intrinsically within the kidney through the upregulation of epithelial VEGF, thus allowing repair to occur. Whether this can and does occur in adult human disease remains to be determined. It is conceivable that the transition from reversible to irreversible injury occurs when the upregulation of VEGF expression occurring in response to simple injury fails to restore adequate circulation for adequate oxygen and nutrients to be provided.



Persistent or extensive injury may tip the balance away from intrinsic repair mechanisms and towards progressive renal disease and renal failure. Whilst the administration of VEGF appears to be generally beneficial in both

acute and progressive renal disease [53–55], its role in underlying pathophysiological processes has to be further determined before therapeutic agents can be optimally utilised.

## References

- 1 Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF: Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983;219:983–985.
- 2 Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N: Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989;246:1306–1309.
- 3 Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA: The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 1991;266:11947–11954.
- 4 Poltorak Z, Cohen T, Sivan R, Kandelis Y, Spira G, Vlodavsky I, Keshet E, Neufeld G: VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. *J Biol Chem* 1997;272:7151–7158.
- 5 Bates DO, Cui TG, Doughty JM, Winkler M, Sugiono M, Shields JD, Peat D, Gillatt D, Harper SJ: VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res* 2002;62:4123–4131.
- 6 Bacic M, Edwards NA, Merrill MJ: Differential expression of vascular endothelial growth factor (vascular permeability factor) forms in rat tissues. *Growth Factors* 1995;12:11–15.
- 7 Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z: Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* 1999;13:9–22.
- 8 Carmeliet P, Ferreira V, Breier G, Pollefeys S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoek A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A: Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996;380:435–439.
- 9 Drake CJ, Little CD: Exogenous vascular endothelial growth factor induces malformed and hyperfused vessels during embryonic neovascularization. *Proc Natl Acad Sci USA* 1995;92:7657–7661.
- 10 Bates DO, Curry FE: Vascular endothelial growth factor increases microvascular permeability via a  $Ca^{2+}$ -dependent pathway. *Am J Physiol* 1997;273:H687–H694.
- 11 Esser S, Wolburg K, Wolburg H, Breier G, Kurzchalia T, Risau W: Vascular endothelial growth factor induces endothelial fenestrations in vitro. *J Cell Biol* 1998;140:947–959.
- 12 Michel CC, Neal CR: Openings through endothelial cells associated with increased microvascular permeability. *Microcirculation* 1999;6:45–54.
- 13 Chen J, Braet F, Brodsky S, Weinstein T, Romanov V, Noiri E, Goligorsky MS: VEGF-induced mobilization of caveolae and increase in permeability of endothelial cells. *Am J Physiol Cell Physiol* 2002;282:C1053–C1063.
- 14 Fu BM, Shen S: Structural mechanisms of acute VEGF effect on microvessel permeability. *Am J Physiol Heart Circ Physiol* 2003;284:H2124–H2135.
- 15 Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E: Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med* 1995;1:1024–1028.
- 16 Ku DD, Zaleski JK, Liu S, Brock TA: Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. *Am J Physiol* 1993;265:H586–H592.
- 17 Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D: Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* 1996;87:3336–3343.
- 18 Gabrilovich DI, Chen HL, Girgis KR, Cunningham HT, Meny GM, Nadaf S, Kavanaugh D, Carbone DP: Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med* 1996;2:1096–1103.
- 19 Mor F, Quintana FJ, Cohen IR: Angiogenesis-inflammation cross-talk: Vascular endothelial growth factor is secreted by activated T cells and induces Th1 polarization. *J Immunol* 2004;172:4618–4623.
- 20 Brown LF, Berse B, Tognazzi K, Manseau EJ, van de WL, Senger DR, Dvorak HF, Rosen S: Vascular permeability factor mRNA and protein expression in human kidney. *Kidney Int* 1992;42:1457–1461.
- 21 Simon M, Grone HJ, Jöhren O, Kullmer J, Plate KH, Risau W, Fuchs E: Expression of vascular endothelial growth factor and its receptors in human renal ontogenesis and in adult kidney. *Am J Physiol* 1995;268:F240–F250.
- 22 Simon M, Rockl W, Hornig C, Grone EF, Theis H, Weich HA, Fuchs E, Yayon A, Grone HJ: Receptors of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) in fetal and adult human kidney: Localization and [125I]VEGF binding sites. *J Am Soc Nephrol* 1998;9:1032–1044.
- 23 Grone HJ, Simon M, Grone EF: Expression of vascular endothelial growth factor in renal vascular disease and renal allografts. *J Pathol* 1995;177:259–267.
- 24 Uchida K, Uchida S, Nitta K, Yumura W, Marumo F, Nihei H: Glomerular endothelial cells in culture express and secrete vascular endothelial growth factor. *Am J Physiol* 1994;266:F81–F88.
- 25 Monacci WT, Merrill MJ, Oldfield EH: Expression of vascular permeability factor/vascular endothelial growth factor in normal rat tissues. *Am J Physiol* 1993;264:C995–C1002.
- 26 Iijima K, Yoshikawa N, Connolly DT, Nakamura H: Human mesangial cells and peripheral blood mononuclear cells produce vascular permeability factor. *Kidney Int* 1993;44:959–966.
- 27 Gruden G, Thomas S, Burt D, Lane S, Chusney G, Sacks S, Viberti G: Mechanical stretch induces vascular permeability factor in human mesangial cells: mechanisms of signal transduction. *Proc Natl Acad Sci USA* 1997;94:12112–12116.
- 28 Iruela-Arispe L, Gordon K, Hugo C, Duijvestijn AM, Claffey KP, Reilly M, Couser WG, Alpers CE, Johnson RJ: Participation of glomerular endothelial cells in the capillary repair of glomerulonephritis. *Am J Pathol* 1995;147:1715–1727.
- 29 Harper SJ, Xing CY, Whittle C, Parry R, Gillatt D, Peat D, Mathieson PW: Expression of neuropilin-1 by human glomerular epithelial cells in vitro and in vivo. *Clin Sci* 2001;101:439–446.
- 30 Thomas S, Vanuytsel J, Gruden G, Rodriguez V, Burt D, Gnudi L, Hartley B, Viberti G: Vascular endothelial growth factor receptors in human mesangium in vitro and in glomerular disease. *J Am Soc Nephrol* 2000;11:1236–1243.
- 31 Robert B, St John PL, Hyink DP, Abrahamson DR: Evidence that embryonic kidney cells expressing flk-1 are intrinsic, vasculogenic angioblasts. *Am J Physiol* 1996;271:F744–F753.
- 32 Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G: A common precursor for hematopoietic and endothelial cells. *Development* 1998;125:725–732.
- 33 Kitamoto Y, Tokunaga H, Tomita K: Vascular endothelial growth factor is an essential molecule for mouse kidney development: Glomerulogenesis and nephrogenesis. *J Clin Invest* 1997;99:2351–2357.

- 4 Tufro A, Norwood VF, Carey RM, Gomez RA: Vascular endothelial growth factor induces nephrogenesis and vasculogenesis. *J Am Soc Nephrol* 1999;10:2125-2134.
- 5 Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, Gerber HP, Kikkawa Y, Miner JH, Quaggin SE: Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. *J Clin Invest* 2003;111:707-716.
- 6 Robert B, Zhao X, Abrahamson DR: Coexpression of neuropilin-1, Flk1, and VEGF(164) in developing and mature mouse kidney glomeruli. *Am J Physiol Renal Physiol* 2000;279:F275-F282.
- 7 Kitamoto Y, Tokunaga H, Miyamoto K, Tomita K: VEGF is an essential molecule for glomerular structuring. *Nephrol Dial Transplant* 2002;17 (suppl 9):25-27.
- 8 Foster RR, Hole R, Anderson K, Satchell SC, Coward RJ, Mathieson PW, Gillatt DA, Saleem MA, Bates DO, Harper SJ: Functional evidence that vascular endothelial growth factor may act as an autocrine factor on human podocytes. *Am J Physiol Renal Physiol* 2003;284:F1263-F1273.
- 9 Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangell L, Wright BD, Radtke F, Aguet M, Ferrara N: VEGF is required for growth and survival in neonatal mice. *Development* 1999;126:1149-1159.
- 10 Ostendorf T, Kunter U, Eitner F, Loos A, Regele H, Kerjaschki D, Henninger DD, Janjic N, Floege J: VEGF(165) mediates glomerular endothelial repair. *J Clin Invest* 1999;104:913-923.
- 11 Pepper MS, Ferrara N, Orci L, Montesano R: Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. *Biochem Biophys Res Commun* 1991;181:902-906.
- 12 Gabbai FB: Effects of nitric oxide synthase blockers on renal function. *Nephrol Dial Transplant* 2001;16(suppl 1):10-13.
- 13 Sugimoto H, Hamano Y, Charytan D, Cosgrove D, Kieran M, Sudhakar A, Kalluri R: Neutralization of circulating vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1) induces proteinuria. *J Biol Chem* 2003;278:12605-12608.
- 14 Kabbani F, Hurwitz HI, Fehrenbacher L, Meropol NJ, Novotny WF, Lieberman G, Griffing S, Bergsland E: Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. *J Clin Oncol* 2003;21:60-65.
- 15 Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, Epstein FH, Sukhatme VP, Karumanchi SA: Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003;111:649-658.
- 16 Asselbergs FW, de Boer RA, Diercks GF, Langeveld B, Tio RA, de Jong PE, van Veldhuisen DJ, van Gilst WH: Vascular endothelial growth factor: The link between cardiovascular risk factors and microalbuminuria? *Int J Cardiol* 2004;93:211-215.
- 17 Klanke B, Simon M, Rockl W, Weich HA, Stolte H, Grone HJ: Effects of vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) on haemodynamics and permselectivity of the isolated perfused rat kidney. *Nephrol Dial Transplant* 1998;13:875-885.
- 18 Kanellis J, Levidiotis V, Khong T, Cox AJ, Stackner SA, Gilbert RE, Cooper ME, Power DA: A study of VEGF and its receptors in two rat models of proteinuria. *Nephron Physiol* 2004;96:26-36.
- 19 Cha DR, Kim NH, Yoon JW, Jo SK, Cho WY, Kim HK, Won NH: Role of vascular endothelial growth factor in diabetic nephropathy. *Kidney Int Suppl* 2000;77:S104-S112.
- 20 Cooper ME, Vranes D, Youssef S, Stackner SA, Cox AJ, Rizkalla B, Casley DJ, Bach LA, Kelly DJ, Gilbert RE: Increased renal expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in experimental diabetes. *Diabetes* 1999;48:2229-2239.
- 21 Kitamura H, Sugisaki Y, Yamanaka N: Endothelial regeneration during the repair process following Habu-snake venom induced glomerular injury. *Virchows Arch* 1995;427:195-204.
- 22 Shimizu A, Masuda Y, Kitamura H, Ishizaki M, Sugisaki Y, Yamanaka N: Recovery of damaged glomerular capillary network with endothelial cell apoptosis in experimental proliferative glomerulonephritis. *Nephron* 1998;79:206-214.
- 23 Kang DH, Hughes J, Mazzali M, Schreiner GF, Johnson RJ: Impaired angiogenesis in the remnant kidney model. II. Vascular endothelial growth factor administration reduces renal fibrosis and stabilizes renal function. *J Am Soc Nephrol* 2001;12:1448-1457.
- 24 Masuda Y, Shimizu A, Mori T, Ishiwata T, Kitamura H, Ohashi R, Ishizaki M, Asano G, Sugisaki Y, Yamanaka N: Vascular endothelial growth factor enhances glomerular capillary repair and accelerates resolution of experimentally induced glomerulonephritis. *Am J Pathol* 2001;159:599-608.
- 25 Kim YG, Suga SI, Kang DH, Jefferson JA, Mazzali M, Gordon KL, Matsui K, Breiteneder-Geleff S, Shankland SJ, Hughes J, Kerjaschki D, Schreiner GF, Johnson RJ: Vascular endothelial growth factor accelerates renal recovery in experimental thrombotic microangiopathy. *Kidney Int* 2000;58:2390-2399.
- 26 Ohashi R, Kitamura H, Yamanaka N: Peritubular capillary injury during the progression of experimental glomerulonephritis in rats. *J Am Soc Nephrol* 2000;11:47-56.
- 27 Ohashi R, Shimizu A, Masuda Y, Kitamura H, Ishizaki M, Sugisaki Y, Yamanaka N: Peritubular capillary regression during the progression of experimental obstructive nephropathy. *J Am Soc Nephrol* 2002;13:1795-1805.
- 28 Kang DH, Kim YG, Andoh TF, Gordon KL, Suga S, Mazzali M, Jefferson JA, Hughes J, Bennett W, Schreiner GF, Johnson RJ: Post-cyclosporine-mediated hypertension and nephropathy: Amelioration by vascular endothelial growth factor. *Am J Physiol Renal Physiol* 2001;280:F727-F736.
- 29 Honkanen E, von Willebrand E, Koskinen P, Teppo AM, Tornroth T, Ruutu M, Gronhagen-Riska C: Decreased expression of vascular endothelial growth factor in idiopathic membranous glomerulonephritis: Relationships to clinical course. *Am J Kidney Dis* 2003;42:1139-1148.
- 30 Shulman K, Rosen S, Tognazzi K, Manseau EJ, Brown LF: Expression of vascular permeability factor (VPF/VEGF) is altered in many glomerular diseases. *J Am Soc Nephrol* 1996;7:661-666.
- 31 Pilmore HL, Eris JM, Painter DM, Bishop GA, McCaughan GW: Vascular endothelial growth factor expression in human chronic renal allograft rejection. *Transplantation* 1999;67:929-933.
- 32 Brenner BM: Nephron adaptation to renal injury or ablation. *Am J Physiol* 1985;249:F324-F337.
- 33 Horita Y, Miyazaki M, Koji T, Kobayashi N, Shibuya M, Razzaque MS, Cheng M, Ozono Y, Kohno S, Taguchi T: Expression of vascular endothelial growth factor and its receptors in rats with protein-overload nephrosis. *Nephrol Dial Transplant* 1998;13:2519-2528.
- 34 De Vriese AS, Tilton RG, Elger M, Stephan CC, Kriz W, Lameire NH: Antibodies against vascular endothelial growth factor improve early renal dysfunction in experimental diabetes. *J Am Soc Nephrol* 2001;12:993-1000.
- 35 Fan L, Wakayama T, Yokoyama S, Amano O, Iseki S: Downregulation of vascular endothelial growth factor and its receptors in the kidney in rats with puromycin aminonucleoside nephrosis. *Nephron* 2002;90:95-102.
- 36 Kang DH, Anderson S, Kim YG, Mazzali M, Suga S, Jefferson JA, Gordon KL, Oyama TT, Hughes J, Hugo C, Kerjaschki D, Schreiner GF, Johnson RJ: Impaired angiogenesis in the aging kidney: Vascular endothelial growth factor and thrombospondin-1 in renal disease. *Am J Kidney Dis* 2001;37:601-611.
- 37 Webb NJ, Watson CJ, Roberts IS, Bottomley MJ, Jones CA, Lewis MA, Postlethwaite RJ, Brenchley PE: Circulating vascular endothelial growth factor is not increased during relapses of steroid-sensitive nephrotic syndrome. *Kidney Int* 1999;55:1063-1071.
- 38 Bailey E, Bottomley MJ, Westwell S, Pringle JH, Furness PN, Feehally J, Brenchley PE, Harper SJ: Vascular endothelial growth factor mRNA expression in minimal change, membranous, and diabetic nephropathy demonstrated by non-isotopic *in situ* hybridisation. *J Clin Pathol* 1999;52:735-738.
- 39 Kuin A, Kruse JJ, Stewart FA: Proteinuria and vascular changes after renal irradiation: the role of reactive oxygen species (ROS) and vascular endothelial growth factor (Vegf). *Radiat Res* 2003;159:174-181.

## CHAPTER 18

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# Shh Expression in Pulmonary Injury and Disease

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### Abstract

The Hedgehog signalling pathway is crucial for normal vertebrate growth and development. Recent studies would suggest that signalling capability is retained in the post-embryonic organism. Shh signalling has been identified in the adult immune system, participating in CD4<sup>+</sup> T lymphocyte activation. Studies on fibrotic pulmonary disorders have demonstrated Shh in both human and mouse lung restricted to areas of active disease. Acute lung injury has also shown upregulated expression and together this data is highly suggestive for a functional role for Shh signalling in adult lung injury and disease. We propose that hedgehog signalling may contribute to epithelial injury and repair and act as an intermediary in cross-talk between damaged epithelium and the immune/inflammatory system.

### Introduction

Work within our group has identified the up-regulated expression of the Hedgehog (Hh) signalling pathway in Idiopathic Pulmonary Fibrosis (IPF) and murine models of fibrotic lung disease.<sup>1</sup> That Hh signalling may also play a role in lung injury is also suggested by the work of<sup>2,3</sup> in their murine model of acute lung injury, and further confirms a role for hedgehog signalling in post embryonic pulmonary tissues.

Another area of interest to our group is the role of Hh signalling in the peripheral immune system. Active hedgehog signalling has been identified in cells of the peripheral immune system.<sup>1,4-6</sup> Since these immune cells are integral to inflammation and repair in the damaged lung, these observations have led to the emergence of the hedgehog signalling pathway as a possible target for therapeutic intervention in conditions of pulmonary damage and disease. The aim of this chapter is to review divergent avenues of research into the hedgehog signalling pathway and relate them to observations made with this pathway at the pulmonary interface.

### The Hedgehog Pathway

The hedgehog signalling pathway has been reviewed extensively elsewhere in this volume and in recent publications,<sup>7</sup> thus will be covered only briefly in this review. There are three vertebrate hedgehog signalling molecules, Sonic Hedgehog (Shh), Desert Hedgehog (Dhh) and Indian Hedgehog (Ihh). Of these, most studies have concentrated on the Shh signalling pathway. Shh mRNA generates an inactive 45kD precursor protein which auto-catalytically cleaves, and is post-translationally modified to produce a highly hydrophobic cholesterol/palmitate modified Shh signalling molecule. This signals either as a cell surface molecule or is secreted

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*Hedgehog-Gli Signaling and Human Disease*, edited by Ariel Ruiz i Altaba. ©2005 Eurekah.com.



following association with the dispatched molecule. Binding of its receptor Patched (Ptc), releases Ptc mediated inhibition of Smoothened (Smo), a G protein like signalling molecule, allowing Smo to influence gene expression through the GLI zinc finger transcription factors. Signalling upregulates Ptc in an auto-regulatory loop, similar to many developmental pathways.

Studies have shown Shh to be a crucial morphogen in a number of developmental systems, including the limbs, lung, gut, nervous and immune systems (for a review see Ref. 8). It is only relatively recently that a role for the hedgehog signalling pathway has been elucidated in the post embryonic organism.

### Hedgehog Signalling in Normal Pulmonary Tissues

Adult lung tissue contains several cell types, including Type I and II epithelial cells, Clara cells, mesenchymal cells (fibroblasts and endothelium), and resident immune system cells including alveolar and interstitial macrophages. Immunolocalisation studies from our group have demonstrated the expression of Shh protein in the lung is restricted to focal patches of epithelium in areas of injury and repair. The hedgehog receptor Patched (Ptc) is detectable on bronchial and alveolar epithelium and in alveolar macrophages.<sup>1</sup> Watkins and colleagues have also identified normal expression of Ptc in the basal layer of bronchial epithelium.<sup>3</sup>

Continued expression of Ptc in the absence of ligand, might indicate regulation and/or signalling via another pathway.<sup>9</sup> However, data emerging from studies in the gastrointestinal tract would suggest that Hh signalling may continue in tissue, in the apparent absence of immunodetectable ligand.

Gastrointestinal tract Shh protein expression is localised to areas of regeneration, such as the fundic glands of the stomach.<sup>10-13</sup> Initially protein expression appeared to be absent from many areas of the adult mammalian tract such as the oesophagus, small intestine and colon, although some cells in these regions appeared to retain the ability to generate mRNA.<sup>10,11,14</sup> Further to this, many of these areas lacked apparent Ptc expression.<sup>12</sup> However, treatment of mice with cyclopamine, a Smo inhibitor, resulted in marked decreases in small intestine epithelial cell proliferation, as evidenced by BrdU incorporation and PCNA studies, suggesting active signalling.<sup>11</sup>

Of interest is the finding that in the mature colon, cyclopamine inhibition of Hh signalling results in a marked *increase* in colonic epithelial proliferation and reduced differentiation of the colonocytes. This alternate response to that of the small intestine may represent a Hh specific effect, with variation in Ihh/Shh expression, or may simply reflect the presence or absence of another signalling factor or downstream effector in the tissues of the colon.<sup>14</sup>

Explanations for signalling in the absence of ligand include the existence of another pathway upstream of Smo (indeed, Ptc-independent pathways have been postulated previously,<sup>15</sup>) or that Hh signalling continued, but at a concentration below immunohistochemical detection. Recent research would support the latter explanation, for Hh expression has now been immunolocalised to areas of the colon, previously described as negative.<sup>10,12</sup> This was achieved through variations in antibody concentration and technique. Thus, future improvement in antibodies, detection methods and specific inhibitors may further clarify this issue for both the gastrointestinal and pulmonary systems.

### Hedgehog Signalling in Injury and Disease

Whilst the studies of Watkins et al demonstrated bronchiolar epithelial expression of the Shh signalling components,<sup>3</sup> we have demonstrated Shh restricted to type II like cells in the alveoli of patients with IPF.<sup>1</sup> Expression was localised to areas of remodelling epithelia, particularly those areas with underlying fibrosis. Interestingly, whilst an irritant induced murine model of fibrosis illustrated similar localisation, a murine model of allergen induced inflammation showed no such upregulation. Whilst both models illustrated evidence of inflammation, the allergen model lacked progressive fibrosis.<sup>1,16</sup>

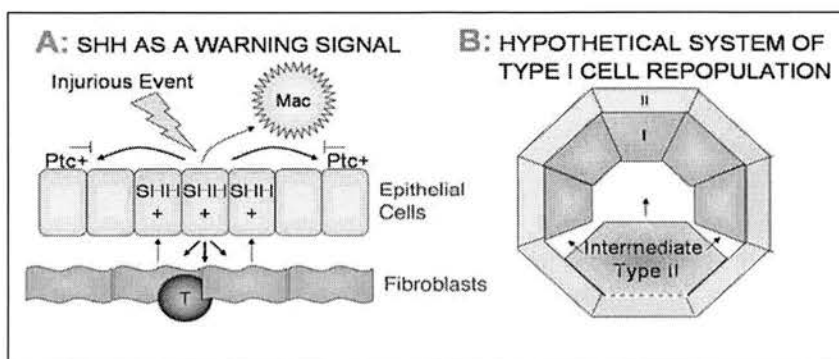


Figure 1. (A) Epithelial cells Shh has the potential to signal to neighboring cells to warn of cellular injury, and generate matrix deposition and proliferation in underlying fibroblasts, preventing transudation in the event of epithelial shedding. Mesenchymal cells produce specific epithelial growth factors such as keratinocyte growth factor (KGF) and fibroblast growth factor 10 (FGF-10). These are upregulated in response to injurious stimuli, resulting in increased epithelial proliferation and rapid reestablishment of an intact epithelial layer, (for a review see Ref. 17). Studies using FGF-10, FGFR2b and Shh knockout mice have recently confirmed Shh to be downstream of FGF-10 signalling.<sup>18</sup> Shh expression on epithelium at sites of fibroblastic proliferation, such as that observed in IPF, may be a consequence of this repair process rather than an instigator of repair. Thus, Shh could be considered a regulatory factor in this instance, given its ability to downregulate FGF-10,<sup>19</sup> (see Fig. 1A).

The upregulation of Shh at sites of epithelial injury comes amongst a plethora of other modulating signals and could therefore represent a downstream marker of a much larger process initiated by the underlying mesenchymal/fibroblastic cells, which play a crucial role in epithelial maintenance. Mesenchymal cells produce specific epithelial growth factors such as keratinocyte growth factor (KGF) and fibroblast growth factor 10 (FGF-10). These are upregulated in response to injurious stimuli, resulting in increased epithelial proliferation and rapid reestablishment of an intact epithelial layer, (for a review see Ref. 17). Studies using FGF-10, FGFR2b and Shh knockout mice have recently confirmed Shh to be downstream of FGF-10 signalling.<sup>18</sup> Shh expression on epithelium at sites of fibroblastic proliferation, such as that observed in IPF, may be a consequence of this repair process rather than an instigator of repair. Thus, Shh could be considered a regulatory factor in this instance, given its ability to downregulate FGF-10,<sup>19</sup> (see Fig. 1A).

Alternatively expression at the alveolar surfaces of IPF patients might relate to a specific element of the disease process, which maybe mirrored in the FITC mouse model, where we observe a similar pattern of expression.<sup>1</sup>

IPF is a chronic fibrotic condition of the lung, associated with inflammation, where median survival after diagnosis is limited to five years. Although without known cause, progression is believed to have an immunological basis, linked in some way with aberrant epithelial mesenchymal interaction.<sup>20</sup> Previous work in this laboratory identified circulating antibodies to a 70-90KD protein in IPF patient serum, which were not observed in normal controls.<sup>21</sup> This protein was later localised to type II cells (Fig. 2), suggesting an autoantigen.<sup>22</sup> Experiments in vivo with a polyclonal antibody raised against the antigen and a human type II epithelial cell line showed upregulated tenascin and TGF- $\beta$  production.<sup>23</sup> Tenascin is an extracellular matrix protein associated with active scar formation, whilst TGF-b can be pro-fibrotic. Both have been well characterised as being present in the lungs of patients with IPF,<sup>24,25</sup> and tenascin has been shown to be localised to areas of active fibroplasias or 'fibroblastic foci'.<sup>23</sup> It will be interesting to discover whether Shh is also upregulated by this interaction, as it would explain its localised expression, at areas of putative antibody interaction, and Shh also has the potential to

be profibrotic. Shh and FGF signalling are well linked in neural systems both pre and postnatally<sup>19,26-28</sup> and hedgehogs have been linked with collagen fibre induction in the neural system.<sup>29</sup> Shh also has substantial cell cycle modulating capabilities<sup>30,31</sup> and participates in well characterised proliferative mesenchymal interactions in the developing lung. Thus the localisation of Shh at fibrotic foci in IPF may also present a causal relationship with fibrosis in the IPF patient.

Similar hypotheses can be drawn from the observations made in the fibrotic FITC mouse model of IPF pathology.<sup>16</sup> Fibrosis and inflammation are believed to be induced in this model through the persistence of the intratracheally instilled irritant FITC, which lodges in the interstitium and binds resident proteins. Antibodies are raised to FITC, and mice develop a pathology similar to that of IPF patients, where Shh expression is localised to sites of damage.<sup>1</sup>

Interestingly, recent work has illustrated a requirement for cell confluence for Shh responsiveness, in a Ptc expressing Shh bioassay.<sup>32,33</sup> Thus, Shh and Ptc expression at a site of proliferative repair, may not automatically indicate that active signalling is occurring. Indeed, the broad expression area of Ptc in the lung might represent a "Shh sponge", to endocytose, in the absence of signalling, free Shh, as described by Torroja and colleagues.<sup>34</sup> However, whilst this may, in part, be true for some expression studies in the lung, work performed in our laboratory and that of others has identified alterations in target gene expression such as Ptc and Gli1, coincident with Shh expression, suggesting that at least some active signalling results from Shh expression.<sup>3</sup>

Shh would appear to share many of its effects with KGF and FGF-10,<sup>30</sup> most notably, stimulation of increased cellular resistance to injury and death.<sup>35,36</sup> Shh exhibits this effect in a number of cell types,<sup>30,37,38</sup> but has yet to be established at the pulmonary interface. Perhaps the best example of this activity is in Parkinson's disease, where exogenous administration of Shh in both a marmoset and mouse model of the disease results in improved locomotion and function.<sup>39,40</sup> Tsuboi and colleagues,<sup>40</sup> citing work by Miao et al.,<sup>38</sup> have suggested that this is likely due to Shh acting as a neuroprotective agent against dopaminergic neuron cell death, which is central to disease progression.

Indeed current research would show that Shh can induce the anti-apoptotic protein Bcl-2 in keratinocytes, and that its presence prevents the apoptosis of neuroepithelial cells.<sup>37</sup> Therefore in a pulmonary situation of injury, such as exposure to environment oxidants, Shh release, along with KGF and FGF-10, may serve to temporarily increase surrounding epithelial cell resilience, and prevent complete denudation of an epithelial surface and the risk of secondary infection, (Fig. 1A).

Sustaining viability of injured cells carries with it the increased probability of populating the epithelium with cells which have incurred genetic damage. Indeed, this may help to explain the higher incidence of small and nonsmall cell lung cancers following epithelial trauma, and the preponderance for the involvement of Shh signalling in these carcinomas.<sup>41</sup>

Upregulation of Hh signalling following injury, can be found in a number of model systems<sup>42,43</sup> have demonstrated the upregulation of Shh and Ptc1 expression in a rodent hind limb ischemia model. Furthermore, the same group demonstrated that the administration of Shh induced robust neovascularisation, enhanced blood flow, and the upregulation of the angiogenic factors, vascular endothelial growth factor (VEGF) and the angiopoietins, Ang1 and Ang2,<sup>43</sup> although any direct association is a matter of conjecture.<sup>44,45</sup> The findings from other groups have been broadly in keeping with those of<sup>43</sup> demonstrating that Shh is capable of inducing VEGF expression<sup>44</sup> and angiogenesis.<sup>45</sup>

Such angiogenic modulation could have important implications at the respiratory surfaces of IPF patients, where we have observed Shh expression in association with fibrotic foci.<sup>1</sup> Shh expression here may represent an attempt by the lung to revascularise and remodel the fibrotic architecture. Alternatively, it may represent a step in the progression of the fibrosis, where VEGF itself is found upregulated with epithelial injury.<sup>46</sup> Perhaps the greatest implication is in

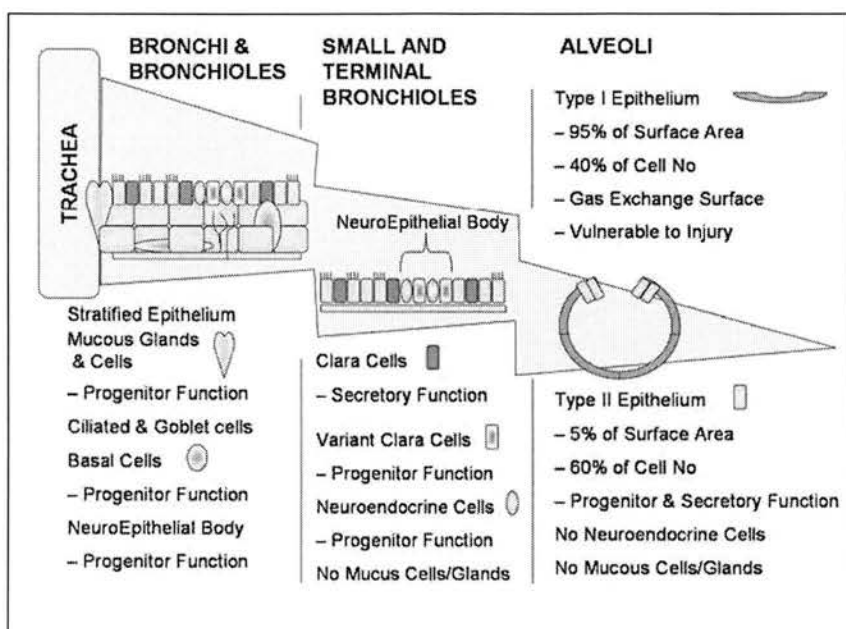


Figure 2.

the neoangiogenic advancement of pulmonary malignancies, which are associated with the expression of Hh pathway components.<sup>3</sup>

### Shh in the Immune System

A functional role for hedgehog signalling in the adult immune system is perhaps not surprising given the well documented role of Shh in lymphoid development and differentiation.<sup>4,9,48</sup> Work in our laboratory has found evidence for expression of Shh and Ptc in both resting and activated murine and human CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, human macrophages and secondary lymphoid tissue,<sup>5,6</sup> both at the mRNA and protein level. This lends support to a possible role for immune-epithelial cell interaction at sites of injury and repair.

It has been postulated that the expression of Hh in a large range of diversified tissues and cell types, might represent a common method of sensing the cellular environment and neighbouring cell health.<sup>49</sup> Given the expression of the Ptc molecule on human macrophages, it may be postulated that Shh expression acts as an advertisement of epithelial damage both to the macrophage, to facilitate attention, and to the adjacent cells as a warning signal for defence and repair initiation (Fig. 1A).

Further to this, Lowrey and coworkers<sup>5,6</sup> have demonstrated that exogenous Shh has the capacity to push sub-optimally activated peripheral CD4<sup>+</sup> T lymphocytes through further cell cycles, whilst addition of a blocking antibody to endogenous Shh results in decreased proliferation. Together these results would suggest that exogenous Shh administration enhances an endogenous stimulatory effect of Shh. Indeed, further work from our group has demonstrated that Shh can act as a costimulatory molecule to T-cells activated via the T cell receptor using

anti-CD3 antibodies (Lowrey et al, unpublished observations). This explains the upregulation of the activation antigens CD25 and CD69, by exogenous Shh, along with the production of Interleukin 2 (IL-2), a T lymphocyte proliferative factor, interleukin 10 (IL-10) and Interferon gamma (IFN- $\gamma$ ). Thus Shh expression at sites of disease and damage has the potential to sustain T lymphocyte mediated immune responses and even to influence the effector function of immune responses, through an IFN- $\gamma$  bias.

Shh has also been shown to upregulate Bcl-2 in T lymphocytes, a step linked with memory T lymphocyte generation,<sup>4</sup> and thus, Shh at sites of injury might also serve to ensure adaptive immunity to damaging antigens, or an exacerbation of autoimmunity. Given that many of the diseases associated with Shh expression contain elements of autoimmunity or immune dysregulation, this crosstalk could have important implications both for the initiation and maintenance of a disease process.

### Hedgehogs and Pulmonary Progenitor Cells

Watkins and colleagues<sup>3</sup> depleted Clara cells from mouse airways via their sensitivity to naphthalene injury. This resulted in a transient peak in Shh and Gli expression 3 days post depletion that coincided with the reestablishment of a complete epithelial barrier. It was suggested that Shh signalling might be involved in this repopulation step, since this peak preceded an increase in the number of pulmonary neuroepithelial cells - a cell type identified as a potential epithelial progenitor.<sup>41,50</sup> Subsequently, Watkins and colleagues identified clusters of epithelial cells expressing Ptc and the neuroendocrine marker, Calcitonin Gene Related Peptide (CGRP), associated with Shh expressing cells in the developing lung.<sup>3</sup> It was suggested that this clustered interaction might continue into the adult pulmonary system, where Shh expression might influence neuroepithelial differentiation.

Presently, progenitor cell phenotypes are characterised via their longevity via nucleotide analogue incorporation, and are defined as label retaining cells. In the mouse these cells are localised to glandular submucosal glands in the distal trachea and bronchi.<sup>51</sup> In the absence of these glands, such as in the bronchioles and alveoli, LRCs occur in localised foci,<sup>52</sup> referred to as neuroepithelial bodies<sup>41</sup> (Fig. 2). These foci are populated by Clara Cell secretory protein (CCSP)-expressing epithelial cells, known as variant Clara cells (vCC), which are naphthalene resistant. Associated with these are CGRP-expressing epithelial cells which show intermediate morphology between a pulmonary Neuroendocrine Cells (PNEC) and a Clara cell<sup>41,50</sup> (Fig. 2). Infiltration of blood borne progenitors in this regenerative process, add a further level of complexity outwith the scope of this chapter, but covered elsewhere.<sup>53</sup>

Label retaining cells undergo hyperplasia in the event of Clara cell death, such as that induced by naphthalene, resulting in the rapid reestablishment of a functional epithelial cell layer. Selective depletion of vCC using a Herpes simplex virus thymidine kinase linkage system, prior to naphthalene injury, results in a failure to repopulate a differentiated ciliated epithelium, despite PNEC hyperplasia.<sup>50</sup> Whether this highlights the CCSP expressing cells as progenitor cells, producers of a signal necessary for PNEC progenitor cell function or as coprogenitors with the PNEC is still an area of conjecture.

As to a potential role for Shh in this process, epithelial coverage is completed at the peak of Shh expression, thus a causative role in nonspecific epithelial proliferation is unlikely, and this is confirmed in our studies where we observe bronchiolar Shh expression restricted to areas of complete epithelial coverage. It is interesting that coverage normally occurs in the absence of differentiation, which typically occurs following reestablishment of epithelial integrity. Given the necessity for confluence for Shh signalling in some cell lines, as mentioned previously, one might speculate that it is in this differentiation step that Shh might play a role.

Reasoning for such speculation arises through the well characterised progenitor modulating role of Hh in a range of post embryonic systems including T lymphocyte development<sup>47</sup> and haematopoiesis.<sup>54</sup> The work by Bhardwaj in haematopoiesis was notable for it clearly defined the downstream effector of Shh function as BMP-4, where Shh addition to an enriched popu-

lation of human CD34+Lin-CD38- progenitor cells resulted in increased self renewal, albeit in combination with many other growth factors.

An example of Hh mediated progenitor modulation in injury comes indirectly from several sources.<sup>55,56</sup> Pepinsky et al demonstrated that Shh administration can accelerate nerve recovery following sciatic nerve crush injury.<sup>55</sup> A Possible explanation for this response comes from studies by Bambakidos et al, using a rat demyelination model.<sup>56</sup> These authors observed increased proliferation of nestin+ve stem cell like progenitors in areas of chemical demyelination following direct Shh administration. This suggests that exogenous Shh at a site of injury induces proliferation in stem cell populations, although the specificity of this progenitor proliferation was not addressed in this study.

The manner in which hedgehog signalling might facilitate this stem cell influence has yet to be characterised, but is likely to lie in its ability to regulate differentiation. Evidence in support of such a role comes from studies of fracture repair, in which Ihh may play a role. Following a fracture, pluripotent mesenchymal cells invade and differentiate into osteoblasts, to initiate the production of hard callus comprising bone matrix, and chondrocytes for the formation of the soft callus. A balanced secondary chondrocyte differentiation step facilitates the remodelling of bone until the bone shape is restored.<sup>57,58</sup> Ihh expression is induced by 3 days post fracture persisting past 2 weeks in a murine model, with the greatest level of expression found in chondrocytes undergoing their secondary differentiation step.<sup>59,60</sup> Ihh expression here induces further chondrocyte proliferation, but prevents further chondrocyte differentiation via PTHrP signalling.<sup>61-63</sup> Given the osteoblast upregulation of PTHrP in the presence of rShh shown by Jemtland and colleagues<sup>64</sup> and the osteoblastic lineage bias induced by Ihh,<sup>65</sup> it is likely that in this system. Ihh is central to restricting differential fates of invading pluripotent mesenchymal cells and facilitates an accurate bone remodelling response.

Central to the stem cell maintenance function of Shh observed in haematopoiesis, is its ability to upregulate BMP-4. These molecules are crucial in early lung morphogenesis and thus have substantial signalling potential in the post embryonic lung. Indeed, studies in a mouse model of allergic inflammation have demonstrated an upregulation of BMP's at sites of inflammation, including BMP-4.<sup>66</sup> In-vitro studies would suggest that BMP-4 has anti-proliferative effects in cancer derived cell lines,<sup>67</sup> however these effects can often be contradictory and dependant on the cosignals and cell types present in culture.

In-vivo studies into pulmonary BMP functions are limited by the lethality of BMP-4 knock-outs. However, innovative in vivo studies in this field from the lab of Brigid Hogan and colleagues have demonstrated a potential progenitor modulating function for BMP's in epithelium. Cells exposed to high levels of BMP-4 retained undifferentiated characteristics.<sup>68,69</sup> In light of these studies, were Shh to modulate progenitor function through the BMP's, it would likely be in conjunction with a number of other signals, such as FGF-10. Perhaps in a recapitulation of the process of cell fate designation in the embryonic lung.<sup>68</sup>

## Shh and Type II Epithelial Cells

Studies thus far have focused on roles for Shh expression at the bronchi and bronchiolar levels, however immunohistochemical data suggest that the hedgehog signal might persist in the larger and smaller airway systems. Watkins and colleagues observed a persistence of Ptc signalling in the progenitor cells of the trachea and bronchi, whilst our lab has identified expression of Shh and Ptc in type II like cells of the terminal bronchioles and alveoli, (Fig. 2).

Pulmonary epithelial regeneration occurs rapidly at the alveolar level. Here, in response to the removal of contact inhibition and KGF,<sup>36</sup> type II cells downregulate specific functional machinery, proliferate and spread out to become thinly spread type I cells, specialised for gaseous exchange. This process occurs almost continually as type I cells are highly susceptible to injury by exogenous factors, i.e., pneumotoxic environmental pollutants and by factors involved in both innate and adaptive pathogen clearance in the lung.



Whilst lack of contact inhibition and KGF are triggers for type II differentiation, no maintenance signal for type II cell number has yet been identified. Perhaps Shh has a modulating function here too. Certainly immunolocalisation in IPF patient biopsies has identified Shh upregulation in individual type II like cells amongst adjacent negative cells, with expression isolated to areas of injury.<sup>1</sup>

This makes for an attractive hypothesis. Damage upregulates type II cell expression of Shh, this induces type II proliferation and inhibits differentiation. However, type II cells adjacent to areas of denuded basement membrane have incomplete confluence and thus lose responsiveness to the Shh, allowing them to differentiate and cover the exposed area (Fig. 1B). However, were Shh a maintenance factor in this continual type II–I transition, it might be expected that normal lung might exhibit some limited immunohistochemical reactivity, and this is not observed. Whether this represents true absence of Shh protein, or the limits of immunohistochemical detection, remains to be determined.

### Concluding Comments

The potential for post embryonic recapitulation of developmental signals in injury and disease presents many exciting targets for therapeutic modulation. This is particularly true for the pulmonary system, where the air interface facilitates the direct and rapid delivery of short half life Hh modulating agents to target cells. This will avoid the systemic complications of treatment that might be expected in the treatment of conditions such as multiple sclerosis and Parkinsons disease. Our knowledge of the Hh signalling pathway and its role in development has made great advancements over recent years and has led to the development of many new and exciting ideas which may identify functions for the Hh pathway in post-embryonic systems.

### References

1. Stewart GA, Hoyne GF, Ahmad SA et al. Expression of the developmental Sonic hedgehog (Shh) signalling pathway is up-regulated in chronic lung fibrosis and the Shh receptor patched 1 is present in circulating T lymphocytes. *J Pathol* 2003; 199(4):488-495.
2. Watkins DN, Berman DM, Baylin SB. Hedgehog signaling: progenitor phenotype in small-cell lung cancer. *Cell Cycle* 2003; 2(3):196-198.
3. Watkins DN, Berman DM, Burkholder SG et al. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature* 2003; 422(6929):313-317.
4. Benson RA, Lowrey JA, Lamb JR et al. The Notch and Sonic hedgehog signalling pathways in immunity. *Mol Immunol* 2004; 41(6-7):715-725.
5. Lowrey JA, Stewart GA, Lindey S et al. Sonic hedgehog promotes cell cycle progression in activated peripheral CD4(+) T lymphocytes. *J Immunol* 2002; 169(4):1869-1875.
6. Stewart GA, Lowrey JA, Wakelin SJ et al. Sonic hedgehog signaling modulates activation of and cytokine production by human peripheral CD4+ T cells. *J Immunol* 2002; 169(10):5451-5457.
7. Nybakken K, Perrimon N. Hedgehog signal transduction: Recent findings. *Curr Opin Genet Dev* 2002; 12(5):503-511.
8. McMahon AP, Ingham PW, Tabin CJ. Developmental roles and clinical significance of hedgehog signaling. *Curr Top Dev Biol* 2003; 53:1-114.
9. Agren M, Kogerman P, Kleman MI et al. Expression of the PTCH1 tumor suppressor gene is regulated by alternative promoters and a single functional Gli-binding site. *Gene* 2004; 330:101-114.
10. van den Brink GR, Hardwick JC, Nielsen C et al. Sonic hedgehog expression correlates with fundic gland differentiation in the adult gastrointestinal tract. *Gut* 2002; 51(5):628-633.
11. van den Brink GR, Hardwick JC, Tytgat GN et al. Sonic hedgehog regulates gastric gland morphogenesis in man and mouse. *Gastroenterology* 2001; 121(2):317-328.
12. Dimmler A, Brabletz T, Hlubek F et al. Transcription of sonic hedgehog, a potential factor for gastric morphogenesis and gastric mucosa maintenance, is up-regulated in acidic conditions. *Lab Invest* 2003; 83(12):1829-1837.
13. Ishizuya-Oka A, Ueda S, Inokuchi T et al. Thyroid hormone-induced expression of sonic hedgehog correlates with adult epithelial development during remodeling of the Xenopus stomach and intestine. *Differentiation* 2001; 69(1):27-37.

14. van den Brink GR, Bleuming SA, Hardwick JC et al. Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. *Nat Genet* 2004; 36(3):277-282.
15. Testaz S, Jarov A, Williams KP et al. Sonic hedgehog restricts adhesion and migration of neural crest cells independently of the Patched- Smoothened-Gli signaling pathway. *Proc Natl Acad Sci USA* 2001; 98(22):12521-12526.
16. Roberts SN, Howie SE, Wallace WA et al. A novel model for human interstitial lung disease: Hapten-driven lung fibrosis in rodents. *J Pathol* 1995; 176(3):309-318.
17. Ware LB, Matthay MA. Keratinocyte and hepatocyte growth factors in the lung: Roles in lung development, inflammation, and repair. *Am J Physiol Lung Cell Mol Physiol* 2002; 282(5):L924-L940.
18. Rice R, Spencer-Dene B, Connor EC et al. Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. *J Clin Invest* 2004; 113(12):1692-1700.
19. Chuang PT, Kawcak T, McMahon AP. Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung. *Genes Dev* 2003; 17(3):342-347.
20. Thannickal VJ, Toews GB, White ES et al. Mechanisms of pulmonary fibrosis. *Annu Rev Med* 2004; 55:395-417.
21. Wallace WA, Roberts SN, Caldwell H et al. Circulating antibodies to lung protein(s) in patients with cryptogenic fibrosing alveolitis. *Thorax* 1994; 49(3):218-224.
22. Wallace WA, Schofield JA, Lamb D et al. Localisation of a pulmonary autoantigen in cryptogenic fibrosing alveolitis. *Thorax* 1994; 49(11):1139-1145.
23. Wallace WA, Howie SE. Upregulation of tenascin and TGFbeta production in a type II alveolar epithelial cell line by antibody against a pulmonary auto-antigen. *J Pathol* 2001; 195(2):251-256.
24. Wallace WA, Howie SE, Lamb D et al. Tenascin immunoreactivity in cryptogenic fibrosing alveolitis. *J Pathol* 1995; 175(4):415-420.
25. Khalil N, O'Connor RN, Unruh HW et al. Increased production and immunohistochemical localization of transforming growth factor-beta in idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol* 1991; 5(2):155-162.
26. Wechsler-Reya RJ, Scott MP. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron* 1999; 22(1):103-114.
27. Ruiz IA, Palma V, Dahmane N. Hedgehog-Gli signalling and the growth of the brain. *Nat Rev Neurosci* 2002; 3(1):24-33.
28. Brewster R, Mullor JL, Altaba A. Gli2 functions in FGF signaling during antero-posterior patterning. *Development* 2000; 127(20):4395-4405.
29. Parmantier E, Lynn B, Lawson D et al. Schwann cell-derived Desert hedgehog controls the development of peripheral nerve sheaths. *Neuron* 1999; 23(4):713-724.
30. Fan H, Khavari PA. Sonic hedgehog opposes epithelial cell cycle arrest. *J Cell Biol* 1999; 147(1):71-76.
31. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 1999; 13(12):1501-1512.
32. Ingram WJ, Wicking CA, Grimmond SM et al. Novel genes regulated by Sonic Hedgehog in pluripotent mesenchymal cells. *Oncogene* 2002; 21(53):8196-8205.
33. Taipale J, Chen JK, Cooper MK et al. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature* 2000; 406(6799):1005-1009.
34. Torroja C, Gorfinkel N, Guerrero I. Patched controls the Hedgehog gradient by endocytosis in a dynamin-dependent manner, but this internalization does not play a major role in signal transduction. *Development* 2004; 131(10):2395-2408.
35. Upadhyay D, Bundesmann M, Panduri V et al. Fibroblast growth factor-10 attenuates H2O2-induced alveolar epithelial cell DNA damage: Role of MAPK activation and DNA repair. *Am J Respir Cell Mol Biol* 2004; 31(1):107-113.
36. Oswari J, Matthay MA, Margulies SS. Keratinocyte growth factor reduces alveolar epithelial susceptibility to in vitro mechanical deformation. *Am J Physiol Lung Cell Mol Physiol* 2001; 281(5):L1068-L1077.
37. Thibert C, Teillet MA, Lapointe F et al. Inhibition of neuroepithelial patched-induced apoptosis by sonic hedgehog. *Science* 2003; 301(5634):843-846.
38. Miao N, Wang M, Ott JA et al. Sonic hedgehog promotes the survival of specific CNS neuron populations and protects these cells from toxic insult In vitro. *J Neurosci* 1997; 17(15):5891-5899.
39. Dass B, Irvani MM, Jackson MJ et al. Behavioural and immunohistochemical changes following supranigral administration of sonic hedgehog in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated common marmosets. *Neuroscience* 2002; 114(1):99-109.
40. Tsuboi K, Shults CW. Intrastriatal injection of sonic hedgehog reduces behavioral impairment in a rat model of Parkinson's disease. *Exp Neurol* 2002; 173(1):95-104.

41. Reynolds SD, Giangreco A, Power JH et al. Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. *Am J Pathol* 2000; 156(1):269-278.
42. Pola R, Ling LE, Aprahamian TR et al. Postnatal recapitulation of embryonic hedgehog pathway in response to skeletal muscle ischemia. *Circulation* 2003; 108(4):479-485.
43. Pola R, Ling LE, Silver M et al. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat Med* 2001; 7(6):706-711.
44. Lawson ND, Vogel AM, Weinstein BM. Sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev Cell* 2002; 3(1):127-136.
45. Kanda S, Mochizuki Y, Suematsu T et al. Sonic hedgehog induces capillary morphogenesis by endothelial cells through phosphoinositide 3-kinase. *J Biol Chem* 2003; 278(10):8244-8249.
46. Pham I, Uchida T, Planes C et al. Hypoxia upregulates VEGF expression in alveolar epithelial cells in vitro and in vivo. *Am J Physiol Lung Cell Mol Physiol* 2002; 283(5):L1133-L1142.
47. Outram SV, Varas A, Pepicelli CV et al. Hedgehog signaling regulates differentiation from double-negative to double-positive thymocyte. *Immunity* 2000; 13(2):187-197.
48. Varas A, Hager-Theodorides AL, Sacedon R et al. The role of morphogens in T-cell development. *Trends Immunol* 2003; 24(4):197-206.
49. Altaba A, Stecca B, Sanchez P. Hedgehog—Gli signaling in brain tumors: Stem cells and paradevelopmental programs in cancer. *Cancer Lett* 2004; 204(2):145-157.
50. Reynolds SD, Hong KU, Giangreco A et al. Conditional clara cell ablation reveals a self-renewing progenitor function of pulmonary neuroendocrine cells. *Am J Physiol Lung Cell Mol Physiol* 2000; 278(6):L1256-L1263.
51. Borthwick DW, Shahbazian M, Krantz QT et al. Evidence for stem-cell niches in the tracheal epithelium. *Am J Respir Cell Mol Biol* 2001; 24(6):662-670.
52. Hong KU, Reynolds SD, Giangreco A et al. Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. *Am J Respir Cell Mol Biol* 2001; 24(6):671-681.
53. Giangreco A, Shen H, Reynolds SD et al. Molecular phenotype of airway side population cells. *Am J Physiol Lung Cell Mol Physiol* 2004; 286(4):L624-L630.
54. Bhardwaj G, Murdoch B, Wu D et al. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat Immunol* 2001; 2(2):172-180.
55. Pepinsky RB, Shapiro RI, Wang S et al. Long-acting forms of Sonic hedgehog with improved pharmacokinetic and pharmacodynamic properties are efficacious in a nerve injury model. *J Pharm Sci* 2002; 91(2):371-387.
56. Bambakidis NC, Wang RZ, Franic L et al. Sonic hedgehog-induced neural precursor proliferation after adult rodent spinal cord injury. *J Neurosurg* 2003; 99(1 Suppl):70-75.
57. Bolander ME. Regulation of fracture repair by growth factors. *Proc Soc Exp Biol Med* 1992; 200(2):165-170.
58. Sandberg MM, Aro HT, Vuorio EI. Gene expression during bone repair. *Clin Orthop* 1993; (289):292-312.
59. Ferguson CM, Miclau T, Hu D et al. Common molecular pathways in skeletal morphogenesis and repair. *Ann NY Acad Sci* 1998; 857:33-42.
60. Vortkamp A, Pathi S, Peretti GM et al. Recapitulation of signals regulating embryonic bone formation during postnatal growth and in fracture repair. *Mech Dev* 1998; 71(1-2):65-76.
61. St Jacques B, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev* 1999; 13(16):2072-2086.
62. Lanske B, Karaplis AC, Lee K et al. PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* 1996; 273(5275):663-666.
63. Zou H, Wieser R, Massague J et al. Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev* 1997; 11(17):2191-2203.
64. Jemtland R, Divieti P, Lee K et al. Hedgehog promotes primary osteoblast differentiation and increases PTHrP mRNA expression and iPTHrP secretion. *Bone* 2003; 32(6):611-620.
65. Spinella-Jaegle S, Rawadi G, Kawai S et al. Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation. *J Cell Sci* 2001; 114(Pt 11):2085-2094.
66. Rosendahl A, Pardali E, Speletas M et al. Activation of bone morphogenetic protein/Smad signaling in bronchial epithelial cells during airway inflammation. *Am J Respir Cell Mol Biol* 2002; 27(2):160-169.

67. Buckley S, Shi W, Driscoll B et al. BMP4 signaling induces senescence and modulates the oncogenic phenotype of A549 lung adenocarcinoma cells. *Am J Physiol Lung Cell Mol Physiol* 2004; 286(1):L81-L86.
68. Weaver M, Yingling JM, Dunn NR et al. Bmp signaling regulates proximal-distal differentiation of endoderm in mouse lung development. *Development* 1999; 126(18):4005-4015.
69. Bellusci S, Henderson R, Winnier G et al. Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. *Development* 1996; 122(6):1693-1702.

# Sonic Hedgehog Signaling Modulates Activation of and Cytokine Production by Human Peripheral CD4<sup>+</sup> T Cells<sup>1</sup>

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Sonic hedgehog (Shh) is important in the growth and differentiation of a variety of cell types, including the development of T cells in the thymus. This prompted us to investigate whether Shh signaling is a functional component of the physiological response of human mature CD4<sup>+</sup> T cells following Ag recognition. In this study, we demonstrate that Shh and its receptor Patched (Ptc) are expressed on resting and activated human peripheral CD4<sup>+</sup> T cells. In approximately one-half of the randomly selected, anonymous blood donors tested, exposure of anti CD3/28 Ab activated CD4<sup>+</sup> T cells to the biologically active N terminal Shh peptide increased the transcription of *ptc*, thereby demonstrating that Shh signaling had occurred. Furthermore, the addition of exogenous Shh amplified the production of IL-2, IFN- $\gamma$ , and IL-10 by activated CD4<sup>+</sup> T cells. The synthesis of IL-2 and IFN- $\gamma$ , but not IL-10, by CD4<sup>+</sup> T cells was down-regulated by the addition of neutralizing anti-Shh Ab. Cell surface expression of CD25 and CD69 on activated T cells was up-regulated by exogenous Shh, whereas in the presence of the neutralizing anti-Shh Ab expression it was reduced. Collectively, our findings demonstrate that Shh-mediated signaling is a physiological component of T cell responses, which acts to modulate CD4<sup>+</sup> T cell effector function. *The Journal of Immunology*, 2002, 169: 5451–5457.

**H**edgehog proteins are highly conserved intercellular signaling molecules that function as morphogens in the development, patterning, and cell fate induction in a range of tissues, such as the CNS, limbs, gastrointestinal tract, and lung (e.g., see Refs. 1–4). Originally described in *Drosophila* as a polarity gene (5), three homologs of these proteins have now been identified in vertebrates of which the biological activity of sonic hedgehog (Shh)<sup>3</sup> has been investigated most extensively (1, 2). Shh is synthesized as a precursor protein that is autoproteolyzed to generate an N-terminal domain (Shh-N) in which the biological activity resides and a C-terminal protein that mediates the autoproteolysis (6, 7). The biologically active Shh-N domain, through its interaction with cholesterol at its N and C termini, respectively, can remain membrane associated and function as a short-range signaling molecule interacting with neighboring cells. Furthermore, as a secreted diffusible molecule, Shh may also deliver long-range signals (8–10). Shh interacts with a receptor complex, which is comprised of two multitransmembrane proteins, Patched (Ptc) and Smoothened (Smo) (11, 12). Ptc is the ligand-binding subunit, which in the absence of Shh inhibits Smo signaling. However, once Shh has bound, Smo is derepressed through a conformational change and transduces the Shh signal across the cell membrane,

which is then mediated by the Gli family of zinc finger transcription factors (1–3).

Shh signaling can induce proliferation in a variety of cell types, which include keratinocytes (13), neuronal precursor cells (14), and hemopoietic stem cells (15). Mutations in *Ptc* and *Smo*, which result in constitutive activation of this signaling pathway, have been demonstrated in proliferative diseases, such as basal cell carcinoma (16), further illustrating the ability of Shh signaling to induce proliferation. Components of the Shh pathway have now been detected in thymus, in which they are reported to contribute to T cell development (17). However, while the receptors Ptc and Smo are expressed on thymocytes, Shh was found only to be present on thymic epithelial cells. By neutralizing the activity of Shh, it was observed that the differentiation of thymocytes from the double-negative (CD4<sup>−</sup>CD8<sup>−</sup>) to double-positive (CD4<sup>+</sup>CD8<sup>+</sup>) stage was increased, whereas the addition of Shh arrested thymocyte development at the double-negative stage (17). It has been reported that Shh can also induce proliferation in human hemopoietic stem cells, and, in addition, the presence of transcripts for *shh*, *Ptc*, and *smo* has been demonstrated in mature CD19<sup>+</sup> and CD3<sup>+</sup> cell populations (15). However, information on the effects of Shh signaling on the function of peripheral T cells is limited (18).

The contribution of the Shh signaling pathway in the development and differentiation of the immune system prompted us to determine whether the activation of this pathway can influence the human peripheral CD4<sup>+</sup> T cell repertoire. We report in this study that Shh and Ptc are present on resting and activated peripheral CD4<sup>+</sup> T cells and that *Ptc* expression is increased in approximately one-half of the randomly selected, anonymous blood donors tested, by the addition of exogenous Shh, indicating, therefore, that Shh signaling had occurred in the T cells. The addition of Shh enhanced the expression of CD25 and CD69 and cytokine production, namely IL-2, IL-10, and IFN- $\gamma$ , by CD4<sup>+</sup> T cells activated by anti-CD3 and anti-CD28 Abs. In the absence of exogenous Shh, the addition of a neutralizing anti-Shh Ab, which recognizes an epitope that overlaps the Ptc binding site of Shh and

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Received for publication July 12, 2002. Accepted for publication September 11, 2002.

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This research was supported by grants from the Medical Research Council (MRC), the Wellcome Trust, and the British Lung Foundation. G.A.S. was the recipient of an MRC Clinical Training Fellowship.

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Abbreviations used in this paper: Shh, Sonic hedgehog; Ptc, Patched; Smo, Smoothened; Tm, melting temperature.



us blocks signaling by preventing Shh binding (19–23), reduced all surface expression of CD25 and CD69 on activated T cells. The production of IL-2 and IFN- $\gamma$ , but not IL-10, was inhibited by the anti-Shh Ab. Taken collectively, our results demonstrate that the induction of Shh signaling in peripheral human CD4<sup>+</sup> T cells modulates T cell activation and cytokine production.

## Materials and Methods

### Isolation of human CD4<sup>+</sup> T cells

Human PBMCs were isolated from randomly selected, anonymized, single-donor Buffy coats, obtained from the Blood Transfusion Center (Royal Infirmary of Edinburgh, U.K.), by centrifugal separation over Histopaque 177 (Sigma-Aldrich, Dorset, U.K.). CD4<sup>+</sup> T cells were separated using negative selection affinity columns (R&D Systems, Abingdon, U.K.), according to the manufacturer's instructions. After separation, the T cells were washed and resuspended in RPMI 1640 culture medium supplemented with 5% heat-inactivated human AB serum, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine (Life Technologies, Paisley, U.K.). Purity of the CD4<sup>+</sup> T cell preparations was analyzed by flow cytometry and was consistently >93%.

### Immunocytochemistry

Purified CD4<sup>+</sup> T cells were spun onto Vectabond-treated microscope slides (Vector Laboratories, Peterborough, U.K.) using a Cytospin 3 (Shandon Scientific, Runcorn, Cheshire, U.K.), set at 300 rpm for 3 min. The cells were air dried and fixed with methanol. After blocking endogenous peroxidase in 3% H<sub>2</sub>O<sub>2</sub>, sections were loaded onto a Sequenza (Shandon Scientific). Nonspecific binding was blocked using normal rabbit serum, and endogenous biotin was blocked using Vector blocking kit (Vector Laboratories), according to the manufacturer's instructions. Slides were incubated with the primary Ab diluted in appropriate serum for 30–120 min at room temperature, washed with PBS, and incubated with a biotinylated secondary Ab for 30 min. They were washed again, and Vector RTU ABC (Vector Laboratories) was applied and positive signal was detected by the addition of diaminobenzidine (DAKO U.K., Ely, U.K.), with a hematoxylin counterstain. Primary Abs to the N terminus of Shh (N-19, 5  $\mu$ g/ml, 1/40 dilution; Santa Cruz Biotechnology, Santa Cruz, CA; Insight Biotechnology, Wembley, Middlesex, U.K.) and to the C terminus of Ptc (C-20, 5  $\mu$ g/ml, 1/40 dilution; Santa Cruz Biotechnology) were used, with a biotinylated anti-goat IgG secondary Ab (DAKO). Both anti-Shh and anti-Ptc Abs are completely blocked by the use of the relevant peptide (data not shown). Images were captured using a Progress 3012 camera (Kontron Elektronik, Zurich, Switzerland) through a Zeiss Axioskop microscope (Oberkochen, Germany).

### CD4<sup>+</sup> T cell cultures

Purified CD4<sup>+</sup> T cells ( $5 \times 10^5$ /ml) were stimulated by the combination of immobilized anti-CD3 (1  $\mu$ g/ml) and soluble anti-CD28 (5  $\mu$ g/ml) Abs (BD Pharmingen, San Diego, CA). In experiments in which either exogenous soluble N-Shh peptide or anti-Shh Ab and isotype control was added, this was performed at the initiation of the cultures.

The mouse rShh, a 180-residue N-terminal peptide (R&D Systems), was constituted in filter-sterilized PBS containing 0.2% endotoxin-free BSA, at a final concentration of 50  $\mu$ g/ml.

The neutralizing anti-Shh Ab (5E1; Developmental Studies Hybridoma Bank, Iowa City, IA) (19–23) and the murine IgG1 isotype control (P3  $\times$  Ag8; European Cell Culture Collection, Porton Down, Wiltshire, U.K.) were purified from hybridoma supernatants using protein G columns (Amersham Pharmacia Biotech, Piscataway, NJ). Western blotting confirmed at 5E1, but not the isotype Ab bound to the Shh peptide (data not shown).

### CD4<sup>+</sup> T cell proliferation

CD4<sup>+</sup> T cells were cultured as above in 96-well plates with and without the addition of exogenous Shh or anti-Shh Ab. Proliferation was assessed by the incorporation of methyl tritiated thymidine ([<sup>3</sup>H]TdR; Amersham Pharmacia Biotech). Cultures were pulsed after 48 h of anti-CD3/28 activated with 1  $\mu$ Ci/well of [<sup>3</sup>H]TdR and at 72 h harvested onto glass fiber filters (Wallac, Milton Keynes, U.K.). [<sup>3</sup>H]TdR incorporation was measured using a Betaplate counter (Wallac).

### Flow cytometry

Phenotypic analysis was performed using PE- and FITC-conjugated mAbs specific for CD3, CD4, CD25, CD69, and the appropriate isotype controls (BD Pharmingen) using a BD Biosciences FACSCalibur (BD Immunocytometry Systems, San Jose, CA).

Viable CD4<sup>+</sup> T cells were gated using forward and side scatter.

### Measurement of cytokine production by ELISA

Cytokine production by purified human CD4<sup>+</sup> T cells (IL-2, IL-10, and IFN- $\gamma$ ) was measured using DuoSet ELISA development system kits (R&D Systems), following the manufacturer's protocols.

### Isolation of RNA and RT-PCR

Extraction of total RNA from purified CD4<sup>+</sup> T cells was performed using TRIzol reagent (Life Technologies), as per the manufacturer's recommendations. The RNA pellets were air dried and resuspended in prewarmed nuclease-free H<sub>2</sub>O (Promega, Southampton, U.K.). To remove any contaminating DNA, the RNA was DNase treated. Total RNA from the CD4<sup>+</sup> T cells was transcribed into cDNA using Access RT-PCR kit (Promega), according to the manufacturer's instructions. The PCR program was as follows: 35 cycles of 30 s at 94°C, 1 min at melting temperature (T<sub>m</sub>) annealing temperature, and 2 min at 68°C, followed by a final extension of 7 min at 68°C. This was performed on a PTC-200 Peltier thermal cycler (MJ Research, Massachusetts, MA).

The following primers were used to detect *Ptc*, with a T<sub>m</sub> of 58°C, giving a PCR product of 462 bp: forward primer, CCATGTTCCAGTTA ATGACTC; reverse primer, ACATCATCCACACCAACA.

The following primers were used to detect *Shh*, with a T<sub>m</sub> of 60°C, giving a PCR product of 335 bp: forward primer, CAGCGGTGTACGT GGTGGC; reverse primer, GGAGCGTCGGCAGACCTG.

The demonstration that transcripts for both *Shh* and *Ptc* are present in the human lung epithelial cell line A549 served as a positive control.

### Real-time RT-PCR

Primers and probes were designed using Primer Express software (PE Biosystems, U.K.). RNA samples for real-time PCR were reverse transcribed into cDNA using TaqMan MultiScribe Reverse Transcriptase kit (PE Biosystems), as per the manufacturer's instructions. The thermocycler was programmed as follows: 1 cycle at 25°C for 10 min, 1 cycle at 48°C for 30 min, and 1 cycle at 95°C for 5 min. The probe specifically annealed the template between the PCR primers. A sequence detector (ABI Prism 7700; PE Biosystems) measured amplification of the product. Each sample was run in duplicate in 96-well optical reaction plates (PE Biosystems). A total of 25  $\mu$ l of the PCR was added per well.

The thermocycler was programmed as follows: 40 cycles of 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min. All the values obtained were normalized to 18S rRNA, which was included in the Multiplex PCR with its own set of primers and probe, as an internal endogenous control.

The following primers and probe were used for analysis of *Ptc* expression: forward primer, CGGACGCCGCGATAAG; reverse primer, TTA ATGATGCCATCTGCATCCA; probe, ATCGACATCAGCCAGTT GAC TAAACAGCGTC.

The software then analyzed the data and output a pair of ct values for each sample. Ct is the number of cycles needed to result in a signal crossing at set threshold. Each sample yielded two ct values, one for the gene of interest and one for the 18S housekeeping control. The ct values were then analyzed to give a value representing the relative mRNA levels present for the gene of interest linearly.

### Statistical analysis

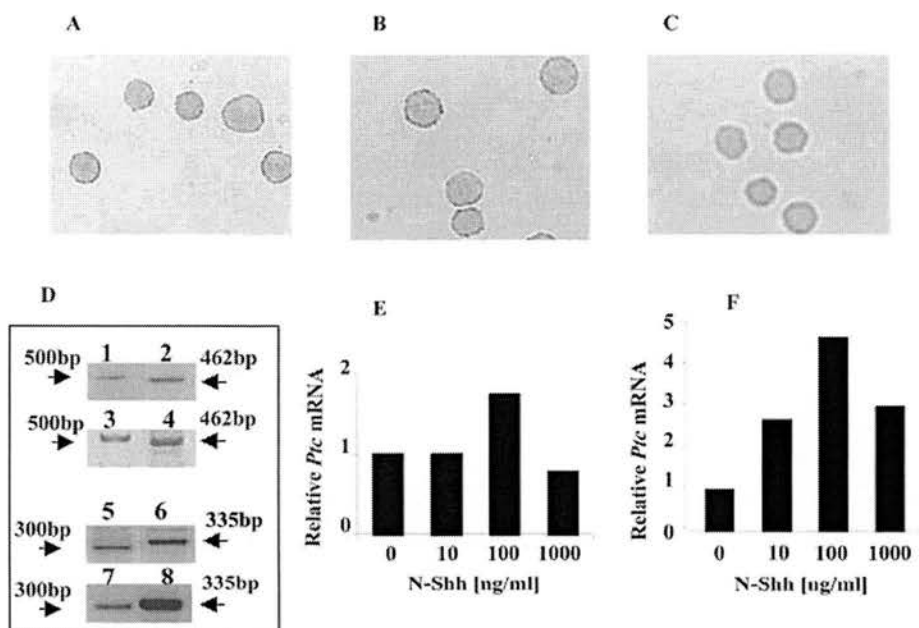
A paired *t* test using a one-tailed *p* value was used to test the significance of the increase in expression of CD25 and CD69. For comparing data from [<sup>3</sup>H]TdR incorporation and cytokine production, a one-way ANOVA was performed with a Tukey-Kramer multiple comparisons post test. Values of *p* < 0.05 were considered significant.

## Results

### Shh signaling pathway is expressed and functional in human peripheral CD4<sup>+</sup> T lymphocytes

To determine the expression of the Ptc and Shh proteins on human peripheral CD4<sup>+</sup> T cells, cytospin preparations of purified CD4<sup>+</sup> T cells were analyzed using immunocytochemistry. Cell surface expression of both Ptc (Fig. 1A) and Shh (Fig. 1B) was observed. Cells stained with normal goat IgG at the same protein concentration were negative (Fig. 1C). The presence of the Shh signaling pathway in CD4<sup>+</sup> T cells was confirmed by RT-PCR because specific mRNAs for both *Ptc* and *Shh* were detected in purified





**FIGURE 1.** Shh signaling pathway is expressed and functional in human peripheral CD4<sup>+</sup> T lymphocytes. Cell membrane expression of Ptc (A) and Shh (B) on cytopins of purified CD4<sup>+</sup> T cells detected by immunocytochemistry with hematoxylin counterstain; cells stained with normal goat IgG are shown in C. Ptc and Shh mRNA (D) expression is shown, as measured by RT-PCR in CD4<sup>+</sup> T cells (track 2, Ptc; track 6, Shh), and in a positive control cell line (A549) derived from a human lung carcinoma (track 4, Ptc; track 8, Shh); DNA ladder bands are shown in tracks 1, 3, 5, and 7. Expression of Ptc mRNA in resting (E) and anti-CD3/28 Ab-activated (F) CD4<sup>+</sup> T cells cultured in the presence of increasing concentrations (10–1000 ng/ml) of N-Shh peptide is shown at 24 h, evaluated by real-time RT-PCR. The real-time reaction is conducted as a multiplex, with 18S RNA as an internal control. Levels of mRNA transcripts are expressed relative to resting (E) and activated CD4<sup>+</sup> T cells (F) cultured in the absence of any N-Shh peptide, which are given a value of 1. Representative experiments of  $n = 5$  are presented.

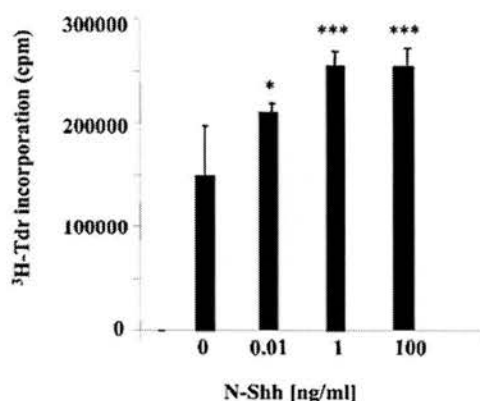
CD4<sup>+</sup>ve T cells and in a control cell line (A549) derived from a human lung carcinoma (Fig. 1D).

It is documented that activation of the Shh pathway results in increased expression of the receptor *Ptc*. Therefore, to determine whether Shh signaling is active in peripheral CD4<sup>+</sup> T cells, the expression of *Ptc* was analyzed by real-time PCR in both resting (Fig. 1E) and anti-CD3/28 Ab-activated (Fig. 1F) CD4<sup>+</sup> T cells in the presence and absence of exogenous Shh. It has previously been reported that a 2-fold increase in gene expression in two or more independent experiments is considered significant (24, 25). The expression of *Ptc*-specific mRNA induced in resting CD4<sup>+</sup> T cells by exogenous Shh is presented relative to the level present in CD4<sup>+</sup> T cells cultured in medium alone at 24 h. We observed that the addition of the N-terminal Shh peptide to resting CD4<sup>+</sup> T cells over a concentration range of 10–1000 ng/ml failed to induce a significant increase in *Ptc* transcripts, although 100 ng/ml had a marginal effect (Fig. 1E). In contrast, relative to anti-CD3/CD28 Ab-activated CD4<sup>+</sup> T cells that were maintained in medium alone, the addition of the exogenous Shh peptide at concentrations of 10 ng/ml or greater resulted in a marked increased in transcription of *ptc* (i.e., >2-fold; Fig. 1F).

#### Shh signaling amplifies CD4<sup>+</sup> T cell effector function

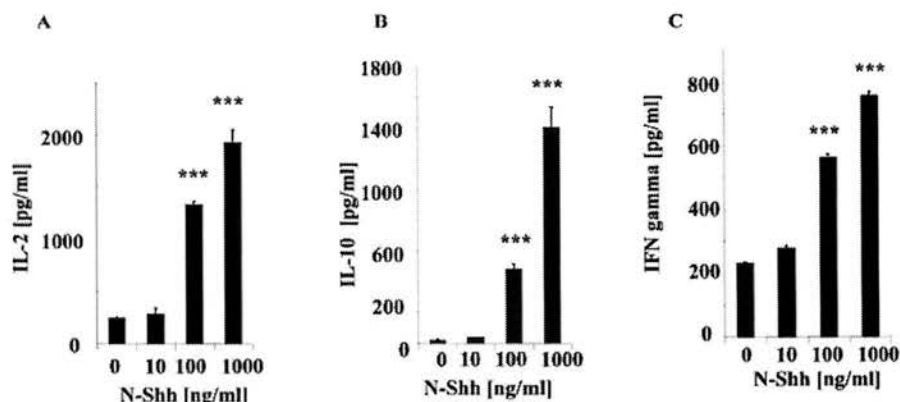
To determine whether Shh signaling contributes to CD4<sup>+</sup> T cell effector function induced following Ag recognition, T cells were stimulated with anti-CD3/CD28 Abs in the presence of exogenous Shh. We observed that the addition of exogenous Shh added at the initiation of the T cell cultures led to a significant enhancement of anti-CD3/CD28 Ab-induced T cell proliferation (Fig. 2), in a dose-dependent manner ( $p < 0.05$  at 0.01 ng/ml of Shh, and  $p < 0.001$  at 1 ng/ml of Shh), but had no effect on resting CD4<sup>+</sup> T cells (data not shown). To further investigate the influence of Shh signaling

on T cell function, cytokine production by activated CD4<sup>+</sup> T cells exposed to increasing concentrations of exogenous N-Shh peptide was determined. We observed that the addition of Shh (>100 ng/ml) significantly ( $p < 0.001$ ) enhanced the levels of IL-2 (Fig. 3A), IL-10 (Fig. 3B), and IFN- $\gamma$  (Fig. 3C). In approximately one-half (6 of 13) of the individuals tested, we noted that the addition of exogenous Shh failed to amplify the proliferation of CD4<sup>+</sup> T cells.



**FIGURE 2.** Exogenous N-Shh enhances the proliferative response of TCR-activated CD4<sup>+</sup> T cells. Purified CD4<sup>+</sup> T cells were activated with immobilized anti-CD3 (1  $\mu$ g/ml) and soluble anti-CD28 (5  $\mu$ g/ml) Abs in the presence of increasing concentrations (0.01–100 ng/ml) of exogenous N-Shh peptide. Proliferation as determined by [<sup>3</sup>H]Tdr incorporation was measured at 72 h and compared with cultures of anti-CD3/28-activated T cells in the absence of Shh. \*\*\*,  $p < 0.001$ ; \*,  $p < 0.05$ . Representative experiment of  $n = 6$  is presented.

**FIGURE 3.** Exogenous N-Shh peptide augments the cytokine production by activated human CD4<sup>+</sup> T cells. Purified CD4<sup>+</sup> T cells were activated with immobilized anti-CD3 (1  $\mu$ g/ml) and soluble anti-CD28 (5  $\mu$ g/ml) Abs in the presence and absence of increasing concentrations (10–1000 ng/ml) of exogenous N-Shh peptide. Supernatants were collected at 2 h, and the levels of IL-2 (A), IL-10 (B), and IFN- $\gamma$  (C) were measured by ELISA and compared with cultures of anti-CD3/28-activated T cells in the absence of Shh. \*\*\*,  $p < 0.001$ . Representative experiment of  $n = 5$  is presented.



In an extension of these studies, the effect of a neutralizing anti-Shh Ab (5E1) on T cell function was investigated. We observed that the addition of the 5E1 at the initiation of the culture period significantly ( $p < 0.01$  for 5  $\mu$ g/ml of 5E1) inhibited the production of IL-2 (Fig. 4A) and IFN- $\gamma$  (Fig. 4C) by anti-CD3/28 Ab-treated CD4<sup>+</sup> T cells in a dose-dependent manner. In contrast, synthesis of IL-10 was unaffected by the addition of 5E1 (Fig. 4B). In the presence of the isotype control at equivalent concentrations, no inhibitory effects on cytokine production were noted (data not shown).

#### Modulation of CD25 and CD69 expression on CD4<sup>+</sup> T cells by exogenous Shh and neutralizing anti-Shh Ab

To further investigate the effects of Shh on CD4<sup>+</sup> T cell activation, the cell surface expression of CD25 and CD69 was analyzed. N-Shh peptide (1000 ng/ml) was added to anti-CD3/28 Ab-activated CD4<sup>+</sup> T cells, and cell surface expression of CD25 and CD69 was measured by FACS analysis at 48 and 72 h after treatment. The FACS profiles for the expression of CD25 and CD69 for activated T cells in the presence and absence of Shh (1000 ng/ml) are shown in Fig. 5, A and B, respectively. Optimum up-regulation of CD25 was seen at 72 h of culture, and of CD69 at 48 h of culture. This was further investigated by titrating the dose of Shh used and observing the increase in these activation markers at their optimum time of induction. The increase in expression was dose dependent (Fig. 5C). In the presence of 5E1 (50  $\mu$ g/ml), the percentage of cells expressing CD25 was reduced, as was the geometric mean fluorescence intensity (Fig. 6A). Similarly, the percentage of T

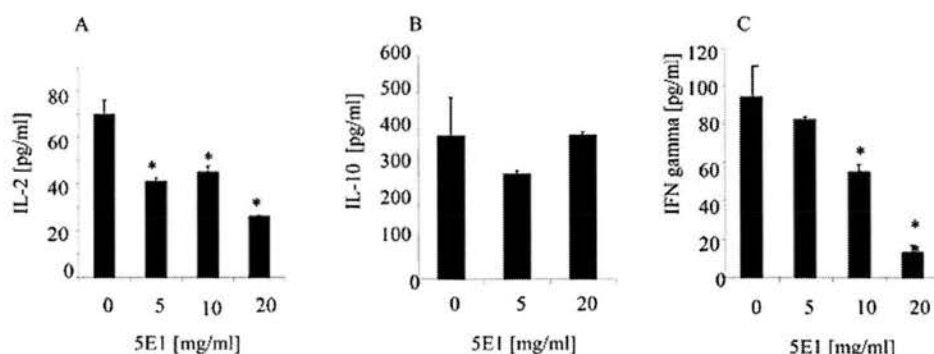
cells expressing CD69 was also increased, as was the geometric mean fluorescence intensity (Fig. 6B).

#### Discussion

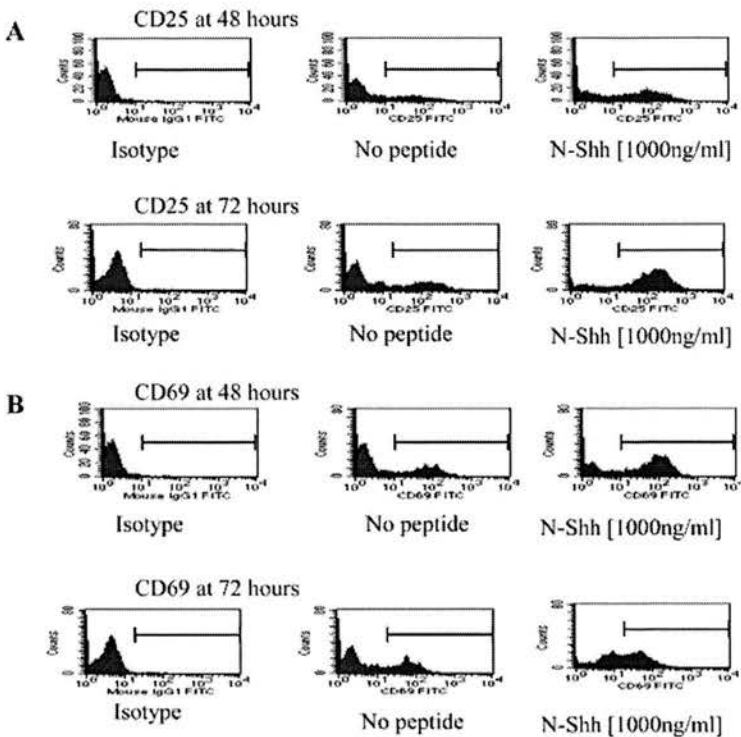
In this study, we have demonstrated that Shh and its receptor Ptc are expressed on human peripheral CD4<sup>+</sup> T cells. We also report that the addition of exogenous Shh enhances the effector function of activated T cells, in approximately one-half of the randomly selected individuals tested, confirming that the Shh signaling pathway is active in the peripheral immune system and has proinflammatory activity. Furthermore, the ability of a neutralizing anti-Shh Ab to inhibit proliferation, the expression of activation Ags, and cytokine production following TCR-mediated activation suggest that the induction of Shh signaling is a physiological component of peripheral CD4<sup>+</sup> T cell responses.

The recognition of peptide/MHC class II complexes expressed on the cell surface of APCs together with secondary signals, such as ligation of costimulatory receptors, are required for the activation, the expansion, and the induction of effector function of CD4<sup>+</sup> T cells (e.g., reviewed in Ref. 26). The results of this study indicate that one action of Shh on the peripheral immune system is to potentiate TCR and costimulatory receptor-mediated signaling and both amplify clonal expansion and enhance the effector function of CD4<sup>+</sup> T cells.

We have shown that human peripheral CD4<sup>+</sup> T cells express both Shh and Ptc, as determined by immunocytochemistry and RT-PCR. Furthermore, we have noted that both Shh and Ptc are present on human CD8<sup>+</sup> T cells (data not shown). Our findings are



**FIGURE 4.** Neutralizing anti-Shh Ab (5E1) down-regulates cytokine production. Anti-Shh Ab (5E1) was added at increasing concentrations (5–20 mg/ml) at the initiation of cultures of purified CD4<sup>+</sup> T cells activated by immobilized anti-CD3 (1  $\mu$ g/ml) and soluble anti-CD28 (5  $\mu$ g/ml) Abs. Supernatants were collected at 72 h, and the levels of IL-2 (A), IL-10 (B), and IFN- $\gamma$  (C) were measured by ELISA and compared with cultures of anti-CD3/28-activated T cells without added 5E1. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ . Representative experiment of  $n = 3$  is presented.



**FIGURE 5.** Kinetics of modulation of cell surface expression of CD25 and CD69 by N-Shh peptide. Purified CD4<sup>+</sup> T cells were activated with immobilized anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml) Abs in the presence of 1000 ng exogenous N-Shh peptide. Cell surface expression of CD25 (A) and CD69 (B) at 48 and 72 h was compared with that on anti-CD3/28-activated T cells in the absence of N-Shh and to an isotype control. Maximum N-Shh up-regulation of CD69 was observed at 48 h, and of CD25 at 72 h; flow cytometric data showing titration curves for this effect at these time points are summarized in C. Representative experiment of *n* = 6 is presented.

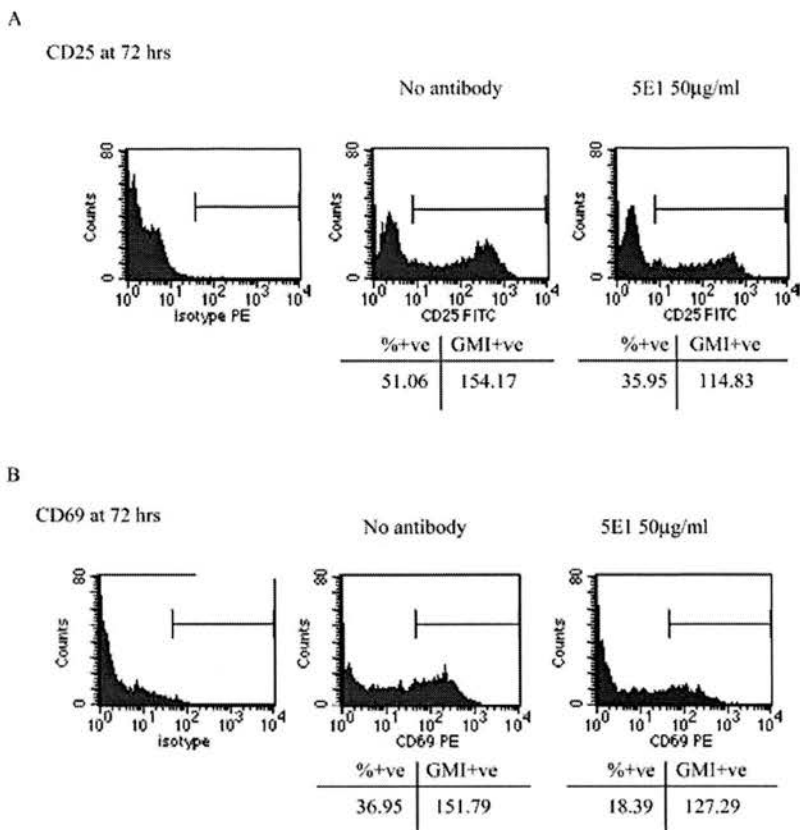
Treatment	% CD69+ve cells [GMI] at 48 hr	% CD25+ve cells [GMI] at 72hr
No peptide	28.32 [65.76]	31.36 [110.84]
N-Shh 10ng/ml	44.98 [59.23]	44.23 [126.97]
N-Shh 100ng/ml	74.35 [89.45]	80.11 [142.25]
N-Shh 1000ng/ml	70.99 [80.29]	84.06 [148.59]

in agreement with and extend those of Bhardwaj et al. (15), who demonstrated using RT-PCR that mature human CD3<sup>+</sup> cells express *Shh*, *Ptc*, and *smo*. In contrast to peripheral T cells, it has been reported that thymocyte populations appear to express only the receptors *Ptc* and *smo* (17), which implies that Shh neither mediates interactions between thymocyte populations nor influences the differentiation and expansion of thymocytes through its autocrine activity. Shh signaling induces the expression of *Ptc* (11) and, therefore, the ability of exogenous Shh to up-regulate *ptc* expression on human CD4<sup>+</sup> T cells activated by anti-CD3/CD28 Ab treatment confirms that this pathway is functional in T cells.

It is well documented that Shh has proliferative effects on a wide range of different cell types, including those of the hemopoietic system (15). In this study, we demonstrate that exogenous Shh enhances TCR-mediated proliferation by CD4<sup>+</sup> T cells. The failure of Shh to induce proliferation or increase expression of *Ptc* on resting CD4<sup>+</sup> T cells suggests that it acts a cofactor, which potentiates TCR-mediated signaling and amplifies clonal expansion. The proliferative activity of Shh is, in part, through its effects on cell cycle (e.g., Refs. 13, 14, 27, 28) and, for example, it has been reported that Shh can increase the number of neuronal precursors in S phase (14). In parallel studies on murine CD4<sup>+</sup> T cells, we have noted that Shh promotes their entry into the proliferative S/G<sub>2</sub> phase of cell cycle (18). Kenney and Rowitch (14) observed that Shh failed to advance quiescent neuronal cells into the cell cycle, which is consistent with our results that Shh has no activity on resting T cells. Furthermore, although it has been reported that Shh can induce *bcl-2* (29), we observed that the addition of Shh failed

to increase survival in activated T cells (data not shown) and, therefore, it is unlikely that enhancement of T cell proliferation by Shh is brought about through a reduction in apoptosis (30–32).

For some individuals, we observed that the addition of exogenous Shh failed to enhance the proliferation response. Mutations in *Ptc* and *smo*, resulting in constitutive activation of Shh signaling, have been reported (33). Thus, polymorphisms that bring about loss or modulation of function are also possible and would provide one explanation for our observations. Furthermore, there is a large body of evidence, which demonstrates that proliferation can be dissociated from other T cell effector functions, such as cytotoxic activity and the production of cytokines (e.g., reviewed in Ref. 33). Therefore, we investigated the ability of Shh to modulate cytokine production by CD4<sup>+</sup> T cells activated by anti-CD3/CD28 Ab treatment. The addition of exogenous Shh to activated T cells increased production of the different cytokines measured in this study, namely IL-2, IL-10, and IFN-γ. There was no evidence that Shh had selective effects on the synthesis of these cytokines. This is in contrast to a previous report in which increasing the threshold of TCR-mediated signaling enhanced IFN-γ production, but had no effect on IL-10 production or T cell proliferation (34). Furthermore, we demonstrate that the addition of exogenous Shh to activated CD4<sup>+</sup> T cells results in the up-regulation of cell surface expression of CD69, implying that the T cells had been further activated by Shh signaling. The expression of CD25 on activated T cells was also enhanced by exogenous Shh. These findings taken together with the observation that Shh enhances the production of IL-2, in part, may be an additional mechanism by which Shh is



**FIGURE 6.** Anti-Shh Ab decreases the expression of CD25 and CD69 on activated CD4<sup>+</sup> T cells. Purified CD4<sup>+</sup> T cells were activated with immobilized anti-CD3 (1 µg/ml) and soluble anti-CD28 (5 µg/ml) Abs in the presence of the anti-Shh Ab (5E1) at 50 µg/ml. Cell surface expression of CD25 (A) and CD69 (B) at 72 h was compared with that on anti-CD3/28-activated T cells in the absence of 5E1. Representative experiment of *n* = 3 is presented.

able to increase the proliferation of activated T cells. It also suggests that Shh signaling does not act by modulating the threshold of TCR signaling (35). We observed that the addition of the neutralizing anti-Shh Ab was able to inhibit the production of IL-2 and IFN-γ, but not IL-10, in contrast to exogenous peptide, which enhanced the synthesis of these cytokines. This suggests that IL-10 production can be regulated by TCR signaling independently of Shh activation. However, one effect of Shh on peripheral T cells appears to be its ability to amplify cytokine production by activated CD4<sup>+</sup> T cells in a nonselective manner.

We demonstrate that the addition of a neutralizing anti-Shh Ab to CD4<sup>+</sup> T cells activated by anti-CD3/28 Ab treatment inhibits T cell proliferation, IL-2 production, and the expression of the activation Ags CD25 and CD69. These findings imply that induction of Shh signaling is a physiological component of T cell activation following the ligation of TCR and costimulatory receptors. Furthermore, this result suggests that Shh produced by CD4<sup>+</sup> T cells themselves in response to TCR-mediated activation functions in an autocrine manner. In the thymus, Shh is expressed on epithelial cells, and Ptc and Smo on the thymocytes (15), suggesting that Shh signaling can also be delivered by APCs. We have also detected Ptc and smo in macrophages (unpublished data). The expression of receptors for Shh on both T cells and APCs would be consistent with the concept that the effector function of both these cell populations can be modulated by Shh signaling. However, the effects of Shh on APC function remain to be determined.

In summary, we show that the Shh signaling is functional in peripheral CD4<sup>+</sup> T cells. Both Shh and its receptor Ptc are expressed on T cells, and they are up-regulated when the T cells are activated by TCR and costimulatory receptor ligation. The functional consequences of this are that T cell proliferation is enhanced and cell surface expression of the activation Ags CD25 and CD69

is increased, as is cytokine production. Furthermore, the ability of a neutralizing Ab to directly inhibit the biological activity of activated T cells suggests that Shh signaling is a physiological component of CD4<sup>+</sup> T cell responses.

## Acknowledgments

We thank June Stewart and Anne Grant for their expert technical assistance.

## References

- Hammerschmidt, M., A. Brook, and A. P. McMahon. 1997. The world according to hedgehog. *Trends Genet.* 13:14.
- Ingham, P. W. 1998. Transducing hedgehog: the story so far. *EMBO J.* 17:3505.
- Theil, T., S. Kaesler, L. Grotewold, J. Bose, and U. Ruther. 1999. Gli genes and limb development. *Cell Tissue Res.* 296:75.
- Van den Brink, G. R., J. C. H. Hardwick, G. N. J. Tytgat, M. A. Brink, F. J. Ten Kate, S. J. H. Van Deventer, and M. P. Peppelenbosch. 2001. Sonic hedgehog regulates gastric gland morphogenesis in man and mouse. *Gastroenterology* 121:317.
- Nusslein-Volhard, C., and E. Wieschaus. 1980. Mutations affecting segment number and polarity in *Drosophila*. *Nature* 287:795.
- Bumcroft, D. A., R. Takada, and A. P. McMahon. 1995. Proteolytic processing yields two secreted forms of sonic hedgehog. *Mol. Cell. Biol.* 15:2294.
- Lee, J. J., S. C. Ekker, D. P. Kessler, J. A. Porter, B. I. Sun, and P. A. Beachy. 1994. Autoproteolysis in hedgehog protein biogenesis. *Science* 266:1528.
- Porter, J. A., D. P. von Kessler, S. C. Ekker, K. E. Young, J. J. Lee, K. Moses, and P. A. Beachy. 1995. The product of hedgehog auto-proteolytic cleavage active in local and long-range signaling. *Nature* 374:363.
- Williams, K. P., P. Rayhorn, G. Chi-Rosso, E. A. Garber, K. L. Strauch, G. S. B. Horan, J. O. Reilly, D. P. Baker, F. R. Taylor, V. Kotliansky, and R. Blake Pepinsky. 1999. Functional antagonists of sonic hedgehog reveal the importance of the N terminus for activity. *J. Cell Sci.* 112:4405.
- Zeng, X., J. A. Goetz, L. M. Suber, W. J. Scott, Jr., C. M. Schreiner, and D. J. Robbins. 2001. A freely diffusible form of sonic hedgehog mediates long-range signaling. *Nature* 411:716.
- Murone, M., A. Rosenthal, and F. J. de Sauvage. 1999. Sonic hedgehog signaling by the patched-smoothed receptor complex. *Curr. Biol.* 9:76.
- Incardona, J. P., J. H. Lee, C. P. Robertson, K. Enga, R. P. Kapur, and H. Roelink. 2000. Receptor-mediated endocytosis of soluble and membrane tethered sonic hedgehog by Patched-1. *Proc. Natl. Acad. Sci. USA* 97:12044.

3. Fan, H., and P. Khavari. 1999. Sonic hedgehog opposes epithelial cell cycle arrest. *J. Cell Biol.* 147:71.
4. Kenney, A. M., and D. H. Rowitch. 2000. Sonic hedgehog promotes G<sub>1</sub> cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. *Mol. Cell Biol.* 20:9055.
5. Bhardwaj, G., B. Murdoch, D. Wu, D. P. Baker, K. P. Williams, K. Chadwick, L. E. Ling, F. N. Karanu, and M. Bhatia. 2001. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat. Immunol.* 2:172.
6. Fujita, E., Y. Khoroku, K. Urabe, T. Tsukahara, M. Y. Momoi, H. Kumagai, T. Takemura, T. Kuroki, and T. Momoi. 1997. Involvement of sonic hedgehog in the cell growth of LK-2 cells, human lung squamous carcinoma cells. *Biochem. Biophys. Res. Commun.* 238:658.
7. Outram, S. V., A. Varas, C. V. Picicelli, and T. Crompton. 2000. Hedgehog signaling regulates differentiation from double-negative to double-positive thymocyte. *Immunity* 13:187.
8. Lowrey, J. A., G. A. Stewart, S. Lindey, G. F. Hoyne, M. J. Dallman, S. E. M. Howie, and J. R. Lamb. 2002. Sonic hedgehog promotes cell cycle progression in activated peripheral CD4<sup>+</sup> T lymphocytes. *J. Immunol.* 169:1869.
9. Ericson, J., S. Morton, A. Kawakami, H. Roelink, and T. M. Jessell. 1996. Two critical periods of sonic hedgehog signaling required for the specification of motor neuron identity. *Cell* 87:661.
10. Zhang, X.-M., and X.-J. Yang. 2001. Temporal and spatial effects of sonic hedgehog signaling in chick eye morphogenesis. *Dev. Biol.* 233:271.
11. Pagan-Westphal, S. M., and C. J. Tabin. 1998. The transfer of left-right positional information during chick embryogenesis. *Cell* 93:25.
12. Fuse, N., T. Maiti, B. Wang, J. A. Porter, T. M. Tanka Hall, D. J. Leahy, and P. A. Beachy. 1999. Sonic hedgehog protein signals not as a hydrolytic enzyme but as an apparent ligand for Patched. *Proc. Natl. Acad. Sci. USA* 96:10992.
13. Pepinsky, R. B., P. Rayhorn, E. S. Day, A. Dergay, K. P. Williams, A. Galdes, F. R. Taylor, P. A. Boraick-Sjodin, and E. A. Garber. 2000. Mapping of sonic hedgehog-receptor interactions by steric interference. *J. Biol. Chem.* 275:10995.
24. Chtanova, T., R. A. Kemp, A. P. Sutherland, F. Ronchese, and C. R. Mackay. 2001. Gene microarrays reveal extensive differential gene expression in both CD4<sup>+</sup> and CD8<sup>+</sup> type 1 and type 2 T cells. *J. Immunol.* 167:3057.
25. Granucci, F., C. Vizzardelli, E. Virzi, M. Rescigno, and P. Ricciardi-Castagnoli. 2001. Transcriptional reprogramming of DCs by differentiation stimuli. *Eur. J. Immunol.* 31:2539.
26. Constant, S. C., and K. Bottomly. 1997. Induction of Th1 and Th2 CD4<sup>+</sup> T cell responses: alternative approaches. *Annu. Rev. Immunol.* 15:297.
27. Duman-Scheel, M., L. Weng, S. Xin, and W. Du. 2002. Hedgehog regulates cell growth and proliferation by inducing cyclin D and cyclin E. *Nature* 417:299.
28. Barnes, E. A., M. Kong, V. Ollendorff, and D. J. Donoghue. 2001. Patched 1 interacts with cyclin B1 to regulate cell cycle progression. *EMBO J.* 20:2214.
29. Fan, H., E. Oro, M. P. Scott, and P. A. Khavari. 1997. Induction of basal cell carcinoma in transgenic human skin expressing sonic hedgehog. *Nat. Med.* 3:788.
30. Nakayama, K. I., K. Nakayama, and I. Negishi. 1993. Disappearance of the lymphoid system in *bcl-2* homozygous mutant chimeric mice. *Science* 261:1584.
31. Strasser, A., A. W. Harris, and S. Cory. 1991. Bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* 67:889.
32. Katsumata, M., R. M. Siegel, D. C. Louie, T. Miyashita, Y. Tsujimoto, P. C. Nowell, M. I. Green, and J. C. Reed. 1992. Differential effects of *bcl-2* on T and B cells in transgenic mice. *Proc. Natl. Acad. Sci. USA* 89:11376.
33. Ruiz i Altaba, A. 1999. Gli proteins and hedgehog signalling in development of cancer. *Trends Genet.* 15:418.
34. Sloan-Lancaster, J., and P. M. Allen. 1996. Altered peptide ligand induced partial T cell activation: molecular mechanisms and roles in T cell biology. *Annu. Rev. Immunol.* 14:1.
35. Verhoef, A., and J. R. Lamb. 2000. Threshold signalling of human Th0 cells in activation and anergy: modulation of effector function by altered TCR ligand. *J. Immunol.* 164:6034.